Functional Characterization of the Wheat Disease Resistance Gene *Lr34* in Heterologous Barley

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Summary

Fungal plant pathogens are a serious threat to global crop production by causing severe yield losses. The ever-growing world population demands a doubling of agricultural production from 2005 until 2050. One strategy to achieve this is the reduction of pathogen-caused yield losses by the investigation and the exploitation of natural plant disease resistance mechanisms. *Lr34* is one of a few quantitative disease resistance genes discovered in wheat and it encodes a full-length ATP-binding cassette (ABC)-transporter. The two predominant alleles of *Lr34* are referred to as *Lr34res* (resistant) and *Lr34sus* (susceptible). Lr34res evolved from Lr34sus by two amino acid polymorphisms resulting in a gain-of-function event (disease resistance). The critical amino acid polymorphisms include a deletion of the phenylalanine residue at position 546 (ΔF546) and a conversion of a tyrosine to a histidine residue at position 634 (T634H). Orthologous *Lr34* genes, all showing the susceptible haplotype regarding the two critical mutations, have been found in rice and *Sorghum* but not in maize, barley and *Brachypodium*. *Lr34res* has been functionally transferred to barley, rice, maize, durum wheat and *Sorghum*. The aim of this thesis was to further characterize the mechanism of *Lr34* using different *Lr34* alleles in barley.

Our results show that expression of *Lr34res* in barley under the control of its native promoter leads to a constitutive reprogramming of the plant metabolism. Multiple pathways involved in basal and induced disease response were up regulated. Further, the hormones jasmonic acid and salicylic acid as well as the secondary metabolites lignin and hordatines reached higher levels in transgenic lines compared to non-transgenic sister lines. We crossed barley lines transgenic for *Lr34res* and *Lr34sus*, respectively. In progeny, *Lr34res* and *Lr34sus* showed interaction on the transcriptional level, leading to lowered fitness costs. This effect was dependant on the expression level of *Lr34res/Lr34sus* in the parent lines. The deletion of F546 in Lr34sus is sufficient to get an Lr34res-associated phenotype. (Chapter B).

The negative pleiotropic effects on growth vigour caused by *Lr34res* in barley can be overcome by using the pathogen-inducible *Hv-Ger4c* promoter to control expression of *Lr34res*. Disease resistance levels were similar to lines transgenic for *Lr34res* under the native wheat promoter in *Hv-Ger4c::Ta-Lr34res* lines while growth parameters were not different from non-transgenic sister lines under glasshouse and near-field conditions. Therefore, a proof-of-concept for barley lines transgenic for *Lr34res* with agronomical potential is provided (Chapter C).

Using artificial *Lr34* alleles we found that the deletion of F546 in the susceptible Lr34 variant, called M2, can be mimicked by the deletions of the neighbouring amino acids I545 and M547, respectively. These results indicate that not all possible gain-of-function mutations occurred naturally. This finding opens the potential to create more resistant elite varieties containing novel resistant *Lr34* alleles by genome editing. Further, we showed that the difference between Lr34res and Lr34sus is based on protein amounts (Chapter D).

Zusammenfassung

Pilzliche Pflanzenpathogene sind eine ernsthafte Bedrohung für den globalen Anbau von Nutzpflanzen, da sie starke Ernteverluste verursachen. Die stetig wachsende Weltbevölkerung erfordert eine Verdopplung der landwirtschaftlichen Produktion von 2005 bis 2050. Eine Strategie zur Erreichung dieses Zieles besteht in der Verminderung krankheitsbedingter Ertragsverluste durch die Erforschung und die Nutzung von natürlichen pflanzlichen Krankeitsresistensmechanismen. *Lr34* ist eines der wenigen in Weizen gefundenen, quantitativen Krankheitsresistenzgene und kodiert für einen „full-length ATP-binding cassette (ABC)-transporter". Die zwei häufigsten Allele werden *Lr34res* (resistent) und *Lr34sus* (anfällig) genannt. Lr34res evolvierte aus Lr34sus durch zwei Unterschiede in der Aminosäurensequenz, welche zu einer neuen Funktion (Vermittlung von Krankheitsresistenz) führte. Die zwei kritischen Unterschiede in der Aminosäurensequenz beinhalten eine Deletion eines Phenylalanins an der Position 546 (ΔF546) und einen Austausch eines Tyrosins durch ein Histidin an der Position 634 (T634H). Orthologe *Lr34* Gene, die aber alle den anfälligen Haplotyp bezüglich der kritschen Mutationen zeigen, wurden in Reis und *Sorghum*, nicht aber in Mais, Gerste oder *Brachypodium* gefunden. *Lr34res* konnte funktional in Gerste, Reis, Mais, Durumweizen und *Sorghum* transformiert werden. Das Ziel dieser Dissertation war es, den Mechanismus der *Lr34*-induzierten Resistenz anhand verschiedener *Lr34* Allele in Gerste zu charakterisieren.

Unsere Resultate zeigen, dass die Expression von *Lr34res* unter der Kontrolle des nativen Weizenpromotors zu einer grundlegenden Veränderung des Metabolismus in Gerste führt. Mehrere, in die basale oder induzierte Immunantwort involvierte, Signalwege waren induziert. In transgenen Linien wurden höhere Mengen der Hormone Jasmonäure und Salicylsäure sowie der sekundären Metaboliten Lignin und Hordatin gefunden als in nicht transgenen Linien. Wir haben Gerstenlinien transgen für *Lr34res* mit Linien transgen für *Lr34sus* gekreuzt. *Lr34res* und *Lr34sus* interagieren auf Transkriptionsebene, was in Abhängigkeit der Expressionsstärke von *Lr34res* und *Lr34sus* in den

Elternpflanzen zu geringeren Fitnesskosten führt. Die Deletion ΔF546 in Lr34sus ist ausreichend um einen Lr34res-assozierten Phänotyp zu generieren (Kapitel B).

Negative pleiotrope Effekte von *Lr34res* auf das Wachstum konnten vermieden werden, wenn die Expression von *Lr34res* durch den pathogen-induzierten *HvGer4c* Promoter gesteuert wurde. In *Hv-Ger4c::Ta-Lr34res* Linien wurde Krankheitsresistenz gleichen Ausmasses wie in Linien mit *Lr34res* unter dem nativen Promoter erreicht. Gleichzeitig blieben die Wachstumsparameter unverändert gegenüber nicht transgener Kontrolllinien. Diese Effekte wurden sowohl im Gewächshaus als auch unter feldnahen Bedingungen beobachtet. Diese Resultate begründen eine konzeptionelle Strategie wie Gerstenlinien mit *Lr34res* von agronomischen Nutzen sein können (Kapitel C).

Mittels künstlicher *Lr34* Allele konnten wir zeigen, dass die Deletion einer benachbarten Aminosäure von F546 (ΔI545 oder ΔM547) ausreichte, um eine anfällige Lr34 Variante, genannt M2, in eine resistente Variante zu überführen. Diese Resultate beweisen, dass nicht alle möglichen funktionsgewinnenden Mutationen in Lr34sus natürlich evolvierten. Weiter wird somit gezeigt, dass die Möglichkeit besteht, neue *Lr34res* Allele mittels “genome-editing“ in Elitesorten zu schaffen. Schliesslich wurde noch gezeigt, dass der Unterschied zwischen Lr34res und Lr34sus auf den jeweiligen Proteinmengen beruht (Kapitel D).

Chapter A: General Introduction

1. Importance of cereal crops

After maize (1,038 million tons) and rice (741 million tons), wheat (729 million tons) and barley (144 million tons) belong to the most important cereal crop species regarding worldwide production (numbers from 2014, FAOstat 2017). All these crop species were domesticated about 10,000 years ago (Garris et al., 2005; Sang, 2009). Maize (*Maize spp.*) is predominantly produced in the United States, Brazil, China and European countries whereas rice (*Oryza sativa*) production is principally located in Asian and African countries. China, India, Europe and the United States are the main contributors for wheat (*Triticum spp.*) production whereas the most important barley (*Hordeum vulgare*) producers include Russia, European countries and Canada (FAOstat 2017). Rice and wheat are mainly cultivated for human nutrition whereas barley and maize mainly serve as animal feed and malt source in brewing industries (Oerke and Dehne, 2004) or as source of industrial products such as starch oil and ethanol (Chaudhary, 2014). The ever growing world population leads to a higher demand for agricultural production. It has been estimated that agricultural production must double until 2050 compared to production values from 2005 (Gerland et al., 2014; Tilman et al., 2011). Productivity, however, is hindered by loss of agricultural land and climate change (Godfray et al., 2010) and recent data demonstrate that the actual yield trends might be insufficient to reach the predicted demand of agricultural production until 2050 (Ray et al., 2013). Cereal crop production is seriously affected by fungal pathogens leading to severe yield losses from 10 to 16% on average (Chakraborty and Newton, 2011; Oerke, 2006). Pathogen resistance against fungicides reduces the amount of available effective pesticides (Borel, 2017). Further, the use of conventional pesticides has become a matter of debate creating a conflict of interest between perceived or real environmental problems and the need for higher production (Lamichhane et al., 2016). Therefore, new approaches to overcome disease-caused yield losses are necessary.

2. The fungal pathogens of wheat and barley

Fungal plant pathogens can be classified according to their lifestyles: biotrophic, necrotrophic and hemi-biotrophic. Biotrophic pathogens grow on living host cells whereas necrotrophic pathogens feed on dead plant material. Hemi-biotrophic pathogens combine a biotrophic phase at infection and feeding, followed by a necrotrophic phase for reproduction. Cereal rusts (caused by species in the order of *Pucciniales*) (Duplessis et al., 2011), powdery mildew (caused by species in the group of *Erysiphales*) (Wiese, 1987) and smut fungi (caused by *Ustilago* species) represent diseases caused by biotrophic fungi. *Zymoseptoria tritici*, the causal agent of septoria tritici blotch, represents an example of a hemi-biotrophic pathogen. Diseases caused by necrotrophic pathogens include leaf blight (caused by *Bipolaris sorokiniana* on wheat and barley) (Sivanesan, 1990; Walters et al., 2012) net blotch (caused by *Pyrenophora teres* on barley*)* (Friis et al., 1991) and scald/leaf blotch (caused by *Rhynchosporium secalis* on barley) (Shipton et al., 1974).

2.1 New threats to wheat production

Wheat resistance breeding is a continuous task due to pathogen evolution and the emergence of new diseases. Wheat blast (caused by *Magnaporthe oryzae*) was first described in 1985 in Brazil (Igarashi S, 1986). The disease is feared due to its potential to lead to yield losses up to 100% (Islam et al., 2016). Recently, the South American lineage of wheat blast spread to Bangladesh indicating that this severe disease might spread further over the world and create a new challenge in wheat production and breeding (Islam et al., 2016; Malaker et al., 2016). So far, *Magnaporthe oryzae* was described as the causing agent for wheat blast (Callaway, 2016; Islam et al., 2016). However, recent phylogenetic studies revealed that wheat blast diseases are caused by multiple *Pyricularia* species (Castroagudin et al., 2016). Although *Pyricularia* and *Magnaporthe* belong to the family of *Magnaporthaceae*, the more precise identification of the wheat blast causing genus *Pyricularia* is of high importance, because the wheat blast pathogen which was found to have spread to Bangladesh

was identified as *Pyricularia graminis-tritici*. Therefore, the newly described species *Pyricularia graminis-tritici* represents a global menace for wheat production.

A new stem rust race was observed in Uganda in 1999 (therefore called *Ug99*) and spread over East Africa into the Middle East within years. Further diversification and adaptation to other wheat lines was later observed (Pretorius et al., 2000; Singh et al., 2015). Although genes mediating resistance against *Ug99* could be found (*Sr28* and *Sr33*) (Babiker et al., 2017; Periyannan et al., 2013), the constant evolution of new or dispersal of existing wheat rust races (wheatrust.org) illustrate the fast pathogen adaptation to modern cultivars. In 2016, a new race of stem rust destroyed tens of thousands of hectares in Sicily. It was the first stem rust epidemic in Europe since the 1950s. Many wheat varieties including hardy durum wheat were shown to be susceptible against this new stem rust race, increasing the probability of pathogen spreading and, therefore, for a return of stem rust in Europe. In addition, two new races of yellow rust were discovered in Europe / North Africa and East Africa / Central Asia, respectively. At least some of these new races are very virulent. In general, warmer autumns and milder winters due to climate change might favor fungal dispersal. Beside rust, powdery mildew belongs to the most important fungal pathogens of cereals (Dean et al., 2012).

2.2 The rust and powdery mildew pathogens of wheat and barley

Rust pathogens in general, include more than 7,000 species belonging to the group of *Pucciniales*, Basidiomycota (Duplessis et al., 2011). Leaf rust (*Puccinia triticina* / *hordei*), stripe rust (*Puccinia* striiformis f.sp. *tritici* / *hordei*) and stem rust (*Puccinia graminis* f.sp. *tritici* / *hordei*) require an alternate host to complete their sexual life cycles. The alternative hosts are *Berberis vulgaris* (for stem and stripe rust) (Jin, 2011) and *Thalictrum speciosissimum* as well as *Ornithogalum spp.* (for leaf rust), (Anikster, 1982; Bolton et al., 2008), respectively. Infection of epidemic scales occurs via wind-driven dispersal of uredospores (Jin et al., 2010; Kolmer, 2005). The fungus survives during

winter in the form of teliospores or as uredia in the straw (Figure 1A, upper part). After landing on the plant leaf surface, uredospores develop a primary germ tube growing towards stomata where an appressorium is created. The fungus forms an infection peg into the substomatal cavity where a substomatal vesicle is formed. Out of the vesicle an infection hyphae is created that forms a haustorial mother cell. The haustorial mother cell penetrates the cell wall and invaginates the mesophyll plasma membrane leading to haustoria. Typical infection symptoms appear as orange-brown pustules containing uredospores 7 to 10 days after infection (Webb and Fellers, 2006) (Figure 1A, lower part). Causal rust pathogens exhibit a high degree of host-specifity. Pathogens causing leaf rust in wheat and barley belong to different species – *Puccinia triticina* and *Puccinia hordei*, respectively (Leppik, 1959). In contrast, stripe and stem rust pathogens belong to the same species that are divided in different *formae speciales* - *Puccinia striiformis* f.sp. *tritici* / *hordei* and *Puccinia graminis* f.sp. *tritici* / *hordei*, respectively (Anikster, 1984; Roelfs et al., 1992).

Powdery mildew species belong to the order of Erysiphales, Ascomycota. In contrast to rust pathogens, no alternative host is required for completion of their the sexual life cycle (Heffer et al., 2006) (Figure 1B, upper part). Epidemic dispersal occurs via wind-driven distribution of conidia. Powdery mildew survives winter as chasmothecia in plant debris. Similarly to leaf rust, the first step of infection occurs via formation of a primary germ tube. In contrast to rust pathogens, the infection peg penetrates the cuticula and haustoria are produced within epidermal cells. The fungus spreads via secondary hyphae that develop from the haustorial structure and infects additional cells of the epidermal layer. Typical infection symptoms are visible as white epiphytic pustules containing conidia 5 to 7 days after infection (Figure 1B, lower part). Similar to rust pathogens, powdery mildew pathogens are characterized by their high host specifity caused by *formae speciales* of one species (e.g. *Blumeria graminis* f.sp *tritici* as the wheat pathogen and *Blumeria graminis* f.sp. *hordei* as the barley pathogen (Inuma et al., 2007; Marchal, 1902)).

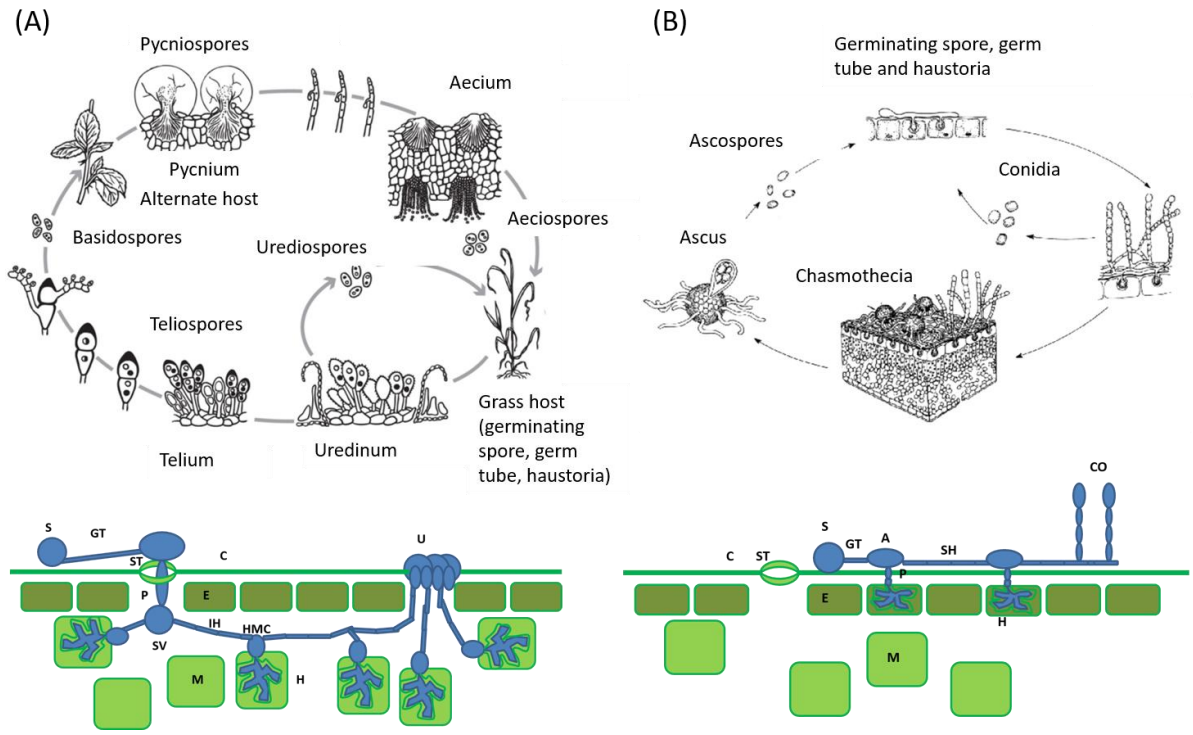

Figure 1: Life cycle of rust and powdery mildew pathogen.

(A) Upper part: Sexual and asexual life cycles of stem rust, adapted from https://www.ars.usda.gov. Lower part: Scheme of infection and asexual reproduction from infection by a spore until the creation of uredia (B) Upper part: Sexual and asexual life cycles of powdery mildew, adapted from http://www.apsnet.org. Lower part: Scheme of infection and asexual reproduction from the infection by a spore until the formation of conidia. S: Spore, GT: Germ tube, A: Appressorium, P: Penetration peg, SH: Secondary hyphae, H: Haustorium, CO: Conidiophore, E: Epidermal cell, M: Mesophyll cell, ST: Stomata, C: Cuticula, SV: Substomatal vesicle IH: Invasive hyphae HMC: Haustorial mother cell, U: Uredia.

3. Plant disease resistance

3.1 Plant resistance mechanisms

Plants are permanently attacked by pathogens. In contrast to animals, plants lack an adaptive immune system. Plant innate immunity is based at least on three distinct mechanisms. The first mechanism is resistance mediated by plasma membrane-localised receptor proteins. The second one mediates race-specific resistance via intracellular immune receptors. The third mechanism is quantitative disease resistance encoded by transporter or kinase proteins (Krattinger and Keller, 2016).

3.1.1 Plasma membrane-localised receptor proteins and PAMP-triggered immunity

Plasma membrane-localised receptor proteins recognize pathogen-associated molecular patterns (PAMPs) (Dodds and Rathjen, 2010; Jones and Dangl, 2006) or plant-derived components that are released to the apoplast upon pathogen penetration such as damage-associated molecular patterns (DAMPs) (Tang et al., 2012). Plasma membrane-localised receptor proteins were described as receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Cook et al., 2015; Dangl et al., 2013; Jones and Dangl, 2006; Thomma et al., 2011). However, not all RLKs are PAMP receptors and not all RLKs receptors recognize conserved structures, as seen for the *Xa21* family that mediates race-specific resistance (Song et al., 1997; Song et al., 1995). PAMP receptors act similar to Toll-like receptors (TLRs) in human innate immunity (Nurnberger et al., 2004). Whereas most of these receptors contain a leucine-rich repeat (LRR) domain, important for ligand binding, also non-LRR domains were shown to recognize pathogen elicitors (Schwessinger and Ronald, 2012). In rice, the RLP (chitin elicitor-binding protein) CEBiP and the RLK chitin receptor elicitor kinase 1 (CERK1) were shown to recognize chitin by a LysM domain (Kaku et al., 2006; Shimizu et al., 2010). Rice *Xa21* is a RLK containing a cytoplasmic kinase domain and an extracellular leucine-rich repeat domain. Xa21 recognizes the pathogen-derived peptide 'Required for activation of Xa21 X' (RaxX), leading to race-specific resistance against *Xanthomonas oryzae* pv. *oryzea* (*Xoo*) (Pruitt et al., 2015; Song et al., 1995). An example for a RLP is Xa26 of rice, mediating resistance against *Xoo* (Sun et al., 2004).

In addition to the membrane-associated receptors described above, some wall-associated receptor-like kinases (WAKs) where shown to mediate disease resistance. As WAKs are known to link the plasma membrane with the cell wall, WAKs might recognize DAMPs released by the plant cell upon fungal penetration. One example is maize *Htn1* encoding an RLK that confers resistance against northern corn leaf blight caused by *Exserohilum turcicum*. *Htn1* mediates quantitative and partial resistance against this adapted pathogen (Hurni et al., 2015). Xa4, a WAK that confers resistance to *Xoo* and modifies cell walls, might act as a DAMP receptor (Krattinger and Keller, 2017).

3.1.2 Intracellular immune receptors recognize the presence of pathogen-effector proteins

Resistance conferred by intracellular immune receptors is based on the recognition of cytoplasmic virulence effectors, small proteins that are secreted by the pathogen to disarm the plant basal immune response. Intracellular immune receptor proteins mostly belong to the conserved family of nucleotide-binding site, leucine-rich repeat receptors (NLRs) (Dodds and Rathjen, 2010). Effector perception of NLRs is associated with programmed cell death of the infected cell, known as hypersensitive response (HR), to prevent pathogen dispersal. This mechanism is very efficient and therefore often called qualitative resistance that is race-specific and therefore prone to be overcome rapidly by pathogens evolution. Examples are the wheat *Pm3* gene that encodes an NLR that confers resistance against powdery mildew by the recognition of AvrPm3 (Bourras et al., 2015). Further, *Lr10* and *Lr21* are wheat genes encoding NLRs that confer resistance against leaf rust (Feuillet et al., 2003; Huang et al., 2003). The rice NLR Pi-ta was shown to confer resistance against rice blast (Huang et al., 2008) and is just one of many cloned *Pi* genes in rice.

3.1.3 Unusual resistance genes that mediate quantitative disease resistance

There are “unusual” resistance genes that do neither code for plasma membrane-associated receptor proteins nor for intracellular immune receptors. Resistance conferred by such genes is often quantitative and partial. Quantitative resistance (*QR*) genes are of special interest for breeding programs as they sometimes confer durable, partial field resistance against most or all races of a pathogen (Ellis et al., 2014; Niks et al., 2015; St Clair, 2010). Durable genes are defined as being effective over a long time period under environmental conditions potentially ideal for the pathogen (Johnson, 1984). One example is the recessive rice blast resistance gene *Pi21*. Interestingly plants with the loss-of-function *pi21* allele showed durable resistance against multiple races of *Magnaporthe oryzae* compared to plant genotypes harboring the functional *Pi21* allele. *Pi21* encodes a proline-rich protein that includes a putative heavy metal-associated domain which is affected in the loss-of-function mutation in the *pi21* allele (Fukuoka et al., 2009). The barley *Mlo*

gene is another example of a recessive resistance gene. There are both *mlo* alleles which were induced as well as naturally occurring alleles that confer durable resistance against several strains of *Blumeria graminis* f.*sp*. *hordei* (Buschges et al., 1997).

QR genes mediating multi-pathogen resistance are per definition effective against multiple pathogen species, whereas broad-spectrum (race non-specific) resistance genes are effective against different races of one species (Krattinger and Keller, 2016). *Xa4* is an example for a race-specific but broad – spectrum resistance gene, because it mediates resistance against most races of *Xoo* in rice (Wang et al., 2001). Another example is wheat *Sr2*, mediating durable partial resistance against all known races of *Puccinia graminis* f. sp. *tritici* (Mago et al., 2014). To date, durable, multi-pathogen *QR* genes were exclusively described in wheat and referred as *Lr46* (=*Yr29/Sr58/Pm39*), *Lr67* (=*Yr46/Sr55/Pm46*) and *Lr34* (=*Yr18/Pm38/Sr57*), respectively. *Lr67* and *Lr34* have been cloned and were described to encode a hexose transporter (Moore et al., 2015) and an ATP-binding cassette (ABC) transporter, respectively (Krattinger et al., 2009).

3.2 The wheat partial, durable, broad-spectrum resistance gene *Lr34*

During the last century, *Lr34* has been introgressed into more than 50% of wheat cultivars used in major breeding programs. So far, no pathogen adaption has been detected, demonstrating its durability (Hoisington et al., 1999; Kolmer et al., 2008). In wheat, *Lr34* is expressed at the adult stage and mediates partial resistance against leaf rust (*Puccinia triticina*), stripe rust (*P. striiformis* f.sp. *tritici*), stem rust (*P. graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) (Ellis et al., 2014; Spielmeyer et al., 2013). Further, *Lr34res* is associated with the senescence-like leaf tip necrosis (LTN) phenotype. In wheat, two predominant *Lr34* alleles, referred as *Lr34res* (resistant) and *Lr34sus* (susceptible) were found on chromosome 7D (Lagudah et al., 2009). *Lr34res* originated from *Lr34sus* by a TTC deletion in exon 11 leading to a deletion of a phenylalanine at residue 546 (ΔF546) and a single nucleotide polymorphism (SNP) C to T in exon 12 causing the conversion from a tyrosine to a histidine at residue 634 (T634H) (Figure 2) (Lagudah et al., 2009). An expressed and

therefore putatively functional (*Lr34-B*) homeolog was found on chromosome 4A. Sequence analysis showed that *Lr34-B* corresponds to the *Lr34sus* haplotype regarding the *Lr34res*-specific nucleotide polymorphisms. Another homeolog was found on chromosome 7A. This homeolog contained several disruptive repetitive elements and was not expressed. Therefore, it was defined as a non functional homeolog (Krattinger et al., 2011). Orthologous *Lr34* genes have been found in rice (*OsABCG50*, 86% identical on protein level), *Sorghum* (*Sb01g016775*, 72% protein identity) but not in barley, maize and *Brachypodium*. However, with regard to two critical polymorphisms, all homeologs in wheat and all orthologs in other grass species showed the susceptible haplotype (Krattinger et al., 2011).

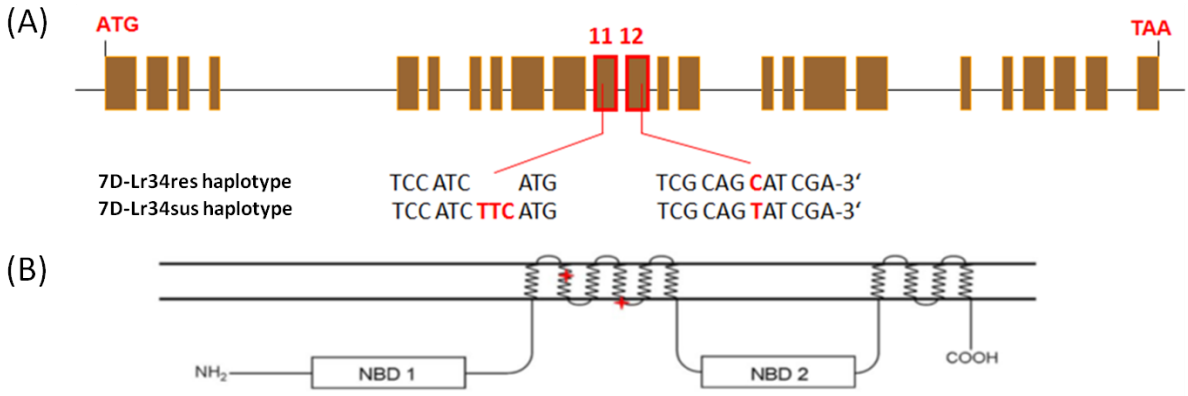

Figure 2: Structure of the *Lr34* gene and protein.

(A) Schematic representation of genomic *Lr34*: Boxes represent exons, lines between boxes indicate introns. (B) Model of protein structure based on prediction tool (wlab.ethz.ch/protter). Horizontal lines indicate membrane. Asterisk show amino acid polymorphisms. NBD= Nucleotide binding domain. Figure adapted from (Krattinger et al., 2013).

3.3.1 Functional transfer of *Lr34res* in other cereals crop species by stable transformation

Lr34res could be functionally transferred into other cereal crop species. In barley, *Lr34res* mediates resistance against barley leaf rust and barley powdery mildew. In contrast to wheat, in barley *Lr34res* is expressed already at the seedling stage although the gene is controlled by the identical promoter. Early expression in barley is leading to LTN in all leaves and impaired growth vigour (Risk et al., 2013). In rice, *Lr34res* was shown to confer resistance against the hemi-biotrophic pathogen *Magnaporthae oryzae*, causing the rice blast disease. Similar to barley, *Lr34res* was expressed already at seedling stage associated with LTN and higher fitness costs, except for one transgenic

event that showed resistance without negative effects (Krattinger et al., 2016). Maize plants transgenic for *Lr34res* showed enhanced resistance against maize common rust (caused by *Puccinia sorghi*) and northern corn leaf blight (NCLB, caused by the hemi-biotrophic pathogen *Exserohilum turcicum*) (Sucher et al., 2017). As in wheat, *Lr34res* was expressed in maize at the adult stage without negative impact on growth vigour (Sucher et al., 2017). *Lr34res* caused enhanced resistance in durum wheat against wheat leaf rust, wheat stripe rust and powdery mildew without negative effects (Rinaldo et al., 2016). Further, *Lr34res* was also functionally transferred to *Sorghum bicolor*, enhancing resistance against *Sorghum* rust (*Puccinia purpurea*) and anthracnose disease (caused by the hemi-biotrophic pathogen *Colletotrichum sublineolum*) with no impact at seedling stage but expression dependant negative effects in adult plants (Schnippenkoetter et al., 2017). In summary *Lr34* functions in all major cereals and provides resistance against a wide range of biotrophic and hemi-biotrophic pathogens. To conclude, wheat *Lr3res* serves as source for partial durable disease resistance in future crop breeding. Although the detailed function of Lr34res is not yet known, the functional transferability to other species indicates a common conserved mechanism within cereal crops. Hulbert et al. (2007) compared transcripts of two pairs of near-isogenic wheat lines for *Lr34* using microarray analysis. Additionally, transcripts of mock-infected plants were compared with plants infected with leaf rust. In wheat, most up-regulated genes in mock-infected plants containing *Lr34* were identified to be involved in pathways induced by abscisic acid (ABA), osmotic stress, cold stress and/or seed maturation. Interestingly, pathogenesis-related (*PR*) genes were not up-regulated in plants harboring *Lr34* compared to their sister lines, although transcript levels of *PR* genes were generally higher in infected plants containing *Lr34* compared to sister lines (Hulbert et al., 2007).

4. Aim of the thesis

The aim of the thesis was to further characterize the mechanism of *Lr34* using barley plants transgenic for different *Lr34* variants. We wanted to characterize changes in the metabolism of barley plants transgenic for *Lr34res* compared to azygous sister lines. Metabolic changes were expected based on the two different observed phenotypes LTN and disease resistance. Therefore, we performed expression analysis and measurement of secondary metabolites in barley lines transgenic for *Lr34res* compared to non-transgenic control lines (Chapter B).

As a next topic we wanted to create barley lines transgenic for *Lr34res* that show disease resistance without negative pleiotropic effects on growth vigour. Doing so, two different strategies to alter *Lr34res* gene expression in barley were tested. The first approach was based on the fact that in wheat *Lr34res* is coexisting with the susceptible *Lr34-B* copy. We wanted to test if the coexistence of a resistant and a susceptible *Lr34* allele in barley changes gene expression of the different *Lr34* alleles compared to lines harboring only one *Lr34* allele. Therefore, we crossed barley lines transgenic for *Lr34res* and *Lr34sus*, respectively. In order to find out if progeny show altered *Lr34res* and/or *Lr34sus* expression levels, progeny were used for gene expression studies. To determine eventual effects on growth vigour, progeny and parental lines were grown to maturity in the glasshouse and compared concerning the development of LTN, above-ground dry weight and grain production (Chapter B). A second strategy to create barley lines with *Lr34res*-mediated disease resistance but no negative pleiotropic effect on growth vigour was to change *Lr34res* gene expression by an inducible promoter distinct from the native wheat promoter. Therefore, *Lr34res* was fused with a pathogen-inducible promoter and stably transformed into barley. To test if the newly created barley lines show potential for agricultural purposes, transgenic plants were assessed for *Lr34res*-mediated disease resistance and growth vigour under semi-field conditions in addition to glasshouse experiments (Chapter C).

Finally, we wanted to find out if both single amino acid polymorphisms (ΔF546 and T634H) are necessary or if only one polymorphism is sufficient to convert a susceptible Lr34 variant into a resistant one. Therefore plants harboring single residue changes were made and analyzed regarding disease resistance (Chapter B). Further, we wanted to test if alternative deletions of amino acids in the region of F546 would convert a susceptible Lr34 variant into a resistant version. Therefore, barley lines transgenic for different artificial *Lr34* alleles were created and analyzed regarding disease resistance (Chapter D).

To get insights into the function of Lr34 at the protein level, barley plants transgenic for different *Lr34* alleles encoding an N-terminal HA-tag were created. Plants transgenic for the corresponding *Lr34* alleles should reveal if natural and artificial alleles lead to different protein levels. Further, we wanted to know if an eventual effect on protein level is correlated with disease resistance (Chapter D).

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Chapter B: The wheat resistance gene *Lr34* results in the constitutive induction of multiple defense pathways in transgenic barley

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Summary

The wheat gene *Lr34* encodes an ABCG-type of transporter which provides durable resistance against multiple pathogens. *Lr34* is functional as a transgene in barley but its mode of action has remained largely unknown both in wheat and barley. Here, we studied gene expression in uninfected barley lines transgenic for *Lr34*. Genes from multiple defense pathways contributing to basal and inducible disease resistance were constitutively active in seedlings and mature leaves. In addition, the hormones jasmonic acid and salicylic acid were induced to high levels and increased levels of lignin as well as hordatines were observed. These results demonstrate a strong, constitutive re-programming of metabolism by *Lr34*. The resistant *Lr34* allele (*Lr34res*) encodes a protein that differs by two amino acid polymorphisms from the susceptible Lr34sus protein. The deletion of a single phenylalanine residue in LR34sus was sufficient to induce the characteristic Lr34res-based responses. Combination of *Lr34res* and *Lr34sus* in the same plant resulted in a reduction of *Lr34res* expression by 8-20 fold when the low-expressing *Lr34res* line BG8 was used as a parent. Crosses with the high-expressing *Lr34res* line BG9 resulted in increase of *Lr34sus* expression by 13–16 fold in progeny that inherited both alleles. These results indicate an interaction of the two *Lr34* alleles on the transcriptional level. Reduction of *Lr34res* expression in BG8 crosses reduced the negative pleiotropic effects of *Lr34res* on barley growth and vigour without compromising disease resistance suggesting that transgenic combination of *Lr34res* and *Lr34sus* can result in agronomical useful resistance.

1. Introduction

Wheat production is severely affected by fungal pathogens, most importantly by the three wheat rust species leaf, stripe and stem rust caused by *Puccinia triticina*, *Puccinia striiformis* and *Puccinia graminis,* respectively. Among these, leaf rust is the most common and widespread, attacking leaves and leaf sheaths of growing wheat plants at most growth stages. Leaf rust can cause 30-70 percent yield losses depending upon disease emergence at the adult plant stage (flag leaf infection) or early infection in seedlings (Huerta-Espino et al., 2011).

The most effective and environmentally sustainable way to control fungal diseases and reduce yield losses is based on new and improved cultivars containing resistance genes. Resistance genes characterized in crop plants can be divided into two different types based on the degree of resistance: *R* genes provide complete but race-specific protection mediated by effector triggered immunity (Jones and Dangl, 2006) whereas quantitative resistance genes (*QR*) provide partial, broad-spectrum, race non-specific and durable protection (Singh et al., 1991; 2005). For these reasons, *QR* genes are more desirable in plant breeding. So far, three durable multi-pathogen rust resistance genes have been genetically identified in wheat: *Lr34* (*Yr18/Pm38/Sr57*) (Dyck *et al*., 1966; McIntosh, 1992; Spielmeyer, 2005), *Lr46* (*Yr29/Pm39/Sr58*) (Lillemo et al., 2007, 2008) and *Lr67* (*Yr46/Pm46/Sr55*) (Herrera-Foessel et al., 2010; Hiebert et al., 2010). These three genes confer durable and partial field resistance against the three wheat rusts and powdery mildew (*Blumeria graminis* f.sp. *tritici*). Of the three genes *Lr34*, *Lr46* and *Lr67*, only *Lr34* has been cloned so far (Krattinger et al., 2009) and has been effectively used in breeding for more than a century.

The *Lr34* gene encodes an ATP binding cassette (ABC) transporter belonging to the ABCG subtype (Krattinger et al., 2009). In addition to resistance to the three rusts and powdery mildew, the *Lr34* locus has further been associated with spot blotch (*Bipolaris sorokiniana)* resistance (Lillemo et al., 2013). There are two expressed and putatively functional homoeologous copies of *Lr34* present in hexaploid wheat on chromosomes 7D and 4A (a translocation from chromosome 7B). The 7D copy of

Lr34 occurs in multiple allelic forms with two predominant alleles, namely *Lr34sus* and *Lr34res* (Dakouri et al., 2010; Krattinger et al., 2011). The resistance-providing protein Lr34res is a gain-of-function variant and differs by only two amino acid changes from other allelic forms (Krattinger et al., 2013, 2011; Dakouri et al., 2010). These two amino acid changes in Lr34res occurred after wheat domestication. On the other hand, only the susceptible haplotype has been found for the second homoeologous *Lr34* copy on chromosome 4A (Krattinger et al., 2013). Comparative analysis in different grass species revealed that there is no *Lr34* ortholog in barley (Krattinger et al., 2011).

Apart from partial multi-pathogen resistance, the presence of *Lr34res* in wheat is also associated with a leaf tip necrosis (LTN) phenotype in the flag leaves of adult plants (Lagudah et al., 2006). This LTN phenotype has been described as the result of a senescence-like process (Krattinger et al., 2009) and depends on *Lr34res* expression levels as well as on environmental conditions (Risk et al., 2012).

Recently, *Lr34res* was functionally transferred into barley where it provided resistance against barley rust *(Puccinia hordei)* and barley powdery mildew (*Blumeria graminis* f.sp. *hordei*). Interestingly, expression of *Lr34res* in barley resulted in multi-pathogen resistance in both seedling and adult plant stages whereas *Lr34res* in wheat only confers resistance at adult plant stage (Risk et al., 2013). No such resistance was observed after transformation of the susceptible allele *Lr34sus* into barley (Risk et al., 2013). The expression of the *Lr34res* transgene in barley resulted in a strong LTN-type of senescence and negative effects on plant growth and fitness, with a lower accumulation of biomass and reduced grain production (Risk et al., 2013).

The molecular mechanism of how Lr34res provides its characteristic durable, multi-pathogen resistance as well as LTN is not known. To expand our knowledge on durable resistance it is essential to understand the molecular details of Lr34res-based resistance. Here, we used barley lines expressing *Lr34res* for a transcriptomics approach and targeted metabolite analysis to identify molecular components in barley that are changed in the presence of *Lr34res*. We observed that *Lr34res* resulted in the induction of various defense pathways even in the absence of pathogen

infection. The combination of *Lr34res* and *Lr34sus* in the same plant resulted in reduced expression levels of *Lr34res* in crosses with *Lr34res* line BG8. Such plants showed lower levels of LTN and increased fitness without compromising disease resistance as compared to plants that only carried the *Lr34res* allele. Progenies of a cross with *Lr34res* line BG9 resulted in an up-regulation of *Lr34sus* when both alleles were combined. These results demonstrate that the negative effects of *Lr34res* in barley can be reduced by a genetic mitigation strategy without compromising disease resistance.

2. Results

We previously reported that the expression of *Lr34res* in transgenic barley resulted in enhanced disease resistance and leaf tip necrosis already in the seedling stage (Risk et al., 2013). The transgenic barley lines provide an optimal system to study the molecular function of Lr34res because (i) in contrast to hexaploid wheat diploid barley shows less genetic redundancy, (ii) apart from *Lr34* the genetic background of transgenic and sister lines is identical and (iii) Lr34res-function can be studied at early seedling stage. We therefore used the Genechip^{R} barley genome array from Affymetrix to identify transcriptional changes in *Lr34res*-containing transgenic barley lines. We chose plants at the T_2 generation of two *Lr34res* transgenic lines, BG8 and BG9 for transcriptomic analysis (Risk et al., 2013). For RNA isolation and microarray hybridization, leaves from two different growth stages were chosen: two-week-old seedlings and ten-week-old mature plants. For seedlings we used the second fully green leaf from plants in which the first leaf just started to develop LTN. For the adult plant stage we used mature leaves that showed LTN that affected about top half of the leaf.

2.1 Differential gene expression in *Lr34res* expressing barley: gene ontology (GO) analysis

In both transgenic lines the analysis of differentially expressed genes revealed major changes in the transcriptome as compared to wild-type in seedlings as well as in mature plants. There were 335 and 1824 probe sets differentially expressed with a log-fold change higher than two (absolute fold

change higher than four) in seedlings and mature leaves, respectively, in at least one of the two transgenic lines (Supplemental tables S1 and S2).

The identities and functional categories of the 50 strongest up-regulated genes at seedling stage and their respective fold changes are listed in Table 1. Many genes related to biotic stress as well as secondary metabolism were up-regulated in *Lr34*-containing barley seedlings. As shown in Supplemental table S1, many of the up-regulated probe sets represent genes related to various *pathogenesis-related* (*PR*) protein genes, such as *PR1* (Contig12046_at), peroxidases (Contig2123_s_at), *chitinases* (Contig4324_s_at), *thaumatins* (Contig2789_at) and genes involved in secondary metabolism such as *carotenoid 9,10(9',10')-cleavage dioxygenase 1-like* (Contig17916_at), *flavanone 3-hydroxylase* (Contig11212_at), *catechol* (*flavanol*) *O-methyltransferase* (Contig17361_at) and *anthranilate N-benzoyltransferase* (Contig15413_at). Apart from these genes, probe sets representing protease inhibitors and transcription factors were up-regulated, for example various WRKY transcription factors (Contig15957_at) and a gene related to the rice *Xa21* type receptor kinase (Contig11866_at) (Table 1 and Supplemental table S1). At the mature plant stage, probe sets representing PR-protein genes and genes involved in secondary metabolism were up-regulated even stronger compared to seedlings with fold change values that were almost 8-10 times higher than the respective fold change at seedling stage. For example *PR1* (Contig12046_at) and *catechol* (*flavanol*) *O-methyltransferase* (Contig17361_at) were 200 and 187 times up-regulated, respectively (Supplemental table S2). To get an idea about the biological function of the up-regulated genes we used the BiNGO visualization tool to produce a visual network of significantly over-represented Gene Ontology (GO) annotations (Maere et al., 2005). Specific and significantly over-represented GO terms were found in the entire GO categories viz. cellular component, biological process and molecular function, in both seedling and mature (Supplemental figure S1) plant stages. The analysis revealed that genes related to cell wall and membrane cellular compartments were significantly over-represented. In the biological process category, there is over-representation of genes related to signaling and response to biotic and endogenous stimuli. These

results indicate that a biotic stress response is induced in *Lr34res*-containing barley plants even in the absence of pathogen attack. In the molecular function categories, genes related to various enzymatic functions such as catalytic, transferase and kinase activities are significantly over-represented. The comparative analysis of expression in seedling leaves with the expression in mature leaves revealed that gene numbers in all these GO categories were increasing as more genes are eventually over-represented in mature leaves.

Table 1: Category wise identities of the 50 strongest up-regulated genes at seedling stage.

Serial No	Probe Set ID	FC[1] BG8	FC BG9	Annotation	Category
1	Contig12046_at	33.8	14.4	PR-1a (Hv-1a)	Defense
2	Contig2123_s_at	23.6	17.3	Peroxidase	Defense
3	Contig4324_at	19.0	4.9	Chitinase II precursor	Defense
4	Contig26496_at	18.7	26.3	Blufensin 2	Defense
5	Contig8896_s_at	14.2	29.2	Cysteine proteinase	Defense
6	Contig2212_s_at	14.1	4.1	Pathogenesis-related protein PRB1-3	Defense
7	Contig4326_s_at	14.1	6.1	Chitinase IV precursor	Defense
8	Contig24926_at	12.7	4.6	Putative LRR serine/threonine protein kinase	Defense
9	Contig17082_at	12.2	8.4	Bowman-Birk type trypsin inhibitor precursor	Defense
10	Contig3776_s_at	11.6	4.0	Putative lipid transfer protein	Defense
11	Contig6519_at	10.2	6.2	Putative pathogen-related protein wir1a	Defense
12	Contig14243_at	9.4	11.2	LRR receptor-like serine/threonine-protein kinase	Defense
13	Contig639_at	9.3	8.1	Pathogenesis-related protein 4	Defense
14	Contig25420_at	9.3	8.1	Immediate-early fungal elicitor protein CMPG1	Defense
15	Contig4406_x_at	9.1	9.5	Pathogenesis-related protein PR-10a	Defense
16	Contig9267_s_at	8.9	9.3	Glucan 1,3-beta-glucosidase precursor	Defense
17	Contig590_s_at	8.4	4.5	PR17c precursor	Defense
18	Contig19684_at	8.1	5.2	Putative hypersensitivity-related (hsr)protein	Defense
19	Contig4402_s_at	7.8	10.5	Pathogenesis-related protein PR-10a	Defense
20	Contig10245_at	7.7	9.9	Disease resistance response protein-related	Defense
21	EBro08_SQ010_O16_at	6.5	5.2	Disease resistance response protein-related	Defense
22	HVSMEm0005L12r2_x_at	6.4	10.1	Putative receptor protein kinase	Defense
23	Contig2163_at	6.4	4.4	Pathogen-induced protein WIR1A	Defense
24	Contig12776_at	5.7	5.9	Glutathione- transferase	Defense
25	Contig11866_at	5.6	5.5	Xa21-rice	Defense
26	Contig17916_at	24.1	7.4	Carotenoid 9,10(9',10')-cleavage dioxygenase 1-like	Secondary metabolism
27	Contig11212_at	22.4	17.7	Flavanone 3-hydroxylase	Secondary metabolism
28	Contig17361_at	20.3	16.9	Catechol (flavanol) O-methyltransferase	Secondary metabolism
29	Contig14651_at	16.6	11.6	Ascorbic acid oxidase	Secondary metabolism
30	Contig26053_at	13.3	10.0	N-methyltransferase	Secondary metabolism
31	Contig14510_at	13.2		L-gulonolactone oxidase-like	Secondary metabolism
32	Contig15413_at	10.2	6.0	Anthranilate N-benzoyltransferase	Secondary metabolism
33	HVSMEb0010O13f2_at	7.8	11.7	Flavin-containing monooxygenase 1	Secondary metabolism
34	HR01G01r_at	6.2	5.7	Putative cytochrome	Secondary metabolism
35	Contig25368_at	6.2	8.0	Putative flavonol glucosyltransferase	Secondary metabolism
36	Contig6082_at	9.9	12.1	Phosphatidylinositol transfer protein PDR17-like	Transporter
37	Contig18758_at	8.4	4.4	Putative potassium transporter	Transporter
38	Contig12753_at	7.3	8.4	Putative ABC transporter (At PDR12)	Transporter
39	Contig22563_at	6.6	6.3	Putative ammonium transporter	Transporter
40	Contig20553_at	6.4	5.8	PDR-like ABC transporter	Transporter
41	S0001000055P18F1_s_at	14.6	20.2	WRKY transcription factor 32	Transcription
42	Contig13288_at	7.2	5.8	Lipoxygenase	Metabolism
43	Contig5469_at	30.8	19.3	Cell number regulator protein	Unknown
44	Contig3783_at	27.3	8.3	Cortical cell-delineating protein-like	Unknown
45	Contig3636_at	10.3	9.6	SERK2 protein	Unknown
46	Contig9408_at	9.9	8.2	LysM receptor-like kinase	Unknown
47	HVSMEm0005L12r2_at	7.2	11.0	Putative receptor protein kinase-like protein	Unknown
48	Contig16619_at	7.0	10.7	Wall-associated kinase 4	Unknown
49	Contig10529_at	6.7	6.0	Sec14 like protein	Unknown
50	Contig8557_at	6.3	4.0	Cysteine-rich repeat secretory protein 55-like	Unknown

2.2 Metabolic pathways affected by the presence of *Lr34res* in transgenic barley

The MapMan omics analysis tool was used to allocate differentially expressed genes to metabolic pathways using barley pathway mappings (Usadel et al., 2009). This analysis revealed that pathways related to biotic stress and secondary metabolism were up-regulated already in seedlings. Over-expression of genes in these pathways became more prominent as plants were maturing and additional pathways were affected such as hormone metabolism, signaling and transport. These changes became even more prominent in the mature leaf stage where many additional, differentially expressed genes were found in pathways related to lipids, flavonoids, phenylpropanoids and phenylalanine and tryptophan metabolism. In contrast, processes and pathways related to photosynthesis, tetrapyrrole synthesis and general RNA metabolism were strongly down-regulated in *Lr34res* containing barley lines (Supplemental figures S2, and S3).

To confirm and study in more detail the induction of the phenylpropanoid pathway and lignin biosynthesis by *Lr34res*, we used qRT-PCR to measure the expression of key genes in the respective pathways. Figure 1a shows the expression of genes involved in lignin biosynthesis. We found that all the key genes involved in monolignol biosynthesis were up-regulated up to 50-fold. For example, the gene encoding phenylalanine ammonia lyase (PAL), the key enzyme of the phenylpropanoid pathway, was up-regulated four fold in seedlings as compared to non-transgenic sibling plants. Figure 1b shows the expression of four genes involved in the biosynthesis of barley defense compounds, namely *anthranilate synthase (AS)*, *anthranilate N-benzoyltransferase (ANB)*, *agmatine coumaroyl transferase (ACT)* and *flavonoid 7-O- methyl transferase (F7OMT)*. All these genes are significantly up-regulated in the transgenic barley leaves.

Several receptor-like kinases genes were up-regulated to various levels in transgenic plants. We confirmed the expression of one of the prominent receptor kinases, a putative homeolog of the rice disease resistance gene *Xa21* by qRT-PCR and found that in both transgenic lines the expression in seedling and mature plant leaves was enhanced ten and forty fold, respectively (Figure 1c). Another

subset of genes for which differential expression was confirmed by qRT-PCR belongs to the biotic stress related bin in MapMan (Supplemental figure S2). We checked the expression of different genes coding for various pathogenesis-related (PR) proteins (Figure 1c). All these *PR* genes were significantly up-regulated in transgenic plants even though there was no infection or disease on the plants. As shown in Figure 1c and Supplemental tables 1 and 2, the up-regulation of various PR-protein genes was exceptionally high, especially for *PR1* (unknown secreted protein). Other *PR* genes encoding proteins such as *PR2* (*β-1,3-glucanase*), *PR3* and *PR4* (*chitinases*), *PR5* (*thaumatin*-like), *PR8* (*chitinase* type III), *PR9* (*peroxidase*) and *PR10* (*ribonuclease-like*) were also up-regulated, suggesting that expression of *Lr34res* induces a large variety of defense proteins involved in a broad spectrum of potential biotic stress responses.

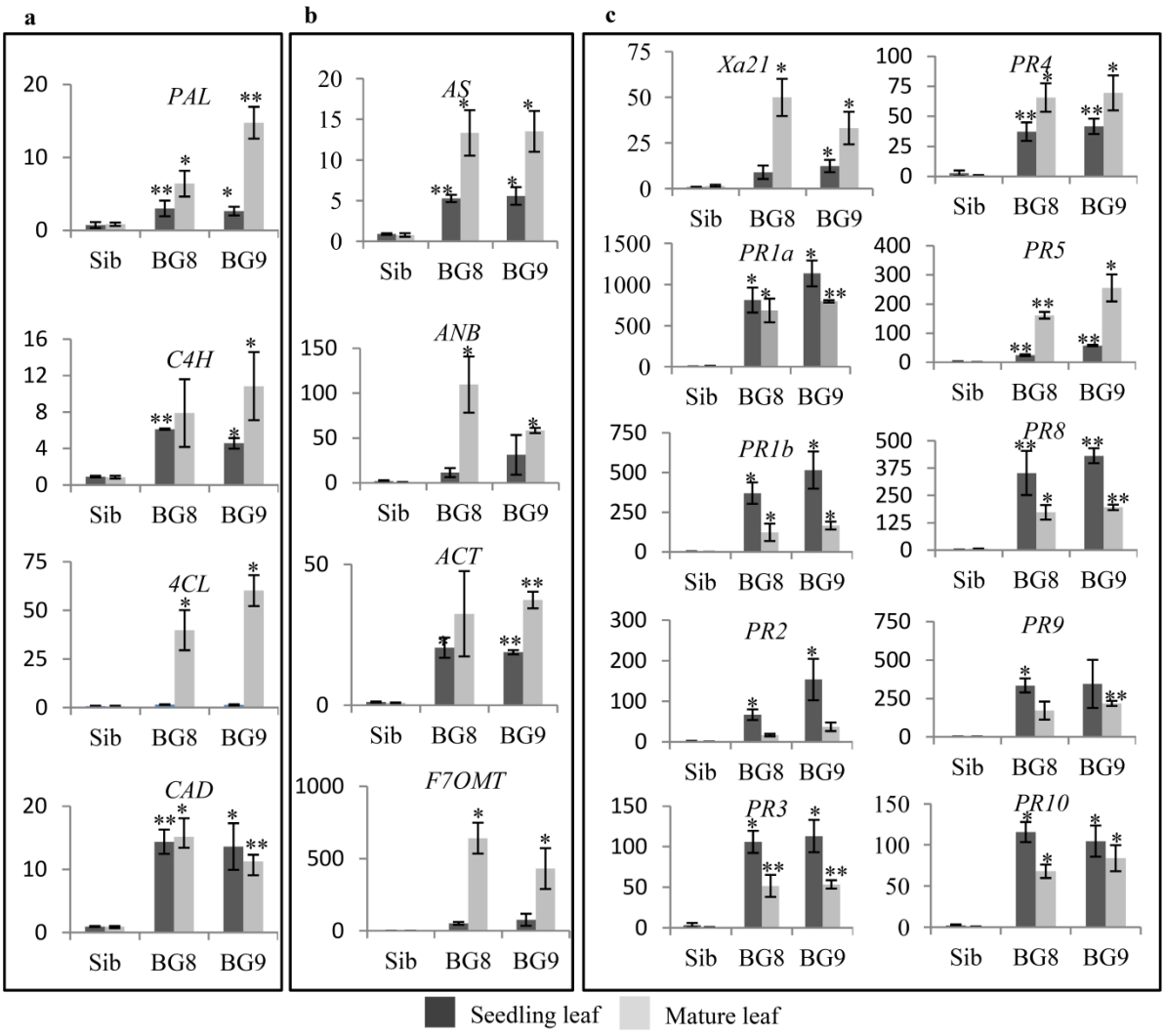

Figure 1: qRT-PCR expression analysis of genes involved in biosynthesis of lignin.

(a) and phytoalexins (b) and PR genes (c). qRT-PCR analyses were performed in Sib plants and plants from two transgenic lines (BG8 and BG9) and two growth stages. Analysis was done in at least three biological replicates and three technical replicates. Values on Y-axis represent relative fold change in comparison to Sib and error bars represent standard error (*=p-value=<0.05; **=p-value=<0.01, calculated by student t-test). (*PAL=phenylalanine ammonia lyase*; *C4H=cinnamate-4-hydroxylate*; *4CL=4-coumarate-CoA ligase*; *CAD=cinnamyl-alcohol dehydrogenase*; *AS=anthranilate synthase*; *ANB=anthranilate N-benzoyltransferase*; *ACT=agmatine coumaroyltransferase*; *F7OMT=flavonoid 7-O- methyltransferase*).

As seen in the overview of the MapMan pathway (Supplemental figure S2) there were several up-regulated genes related to hormone biosynthesis, especially jasmonic acid (JA) biosynthesis. We tested by qRT-PCR the expression of key JA biosynthesis genes, *allene oxide synthase (AOS)*, *allene oxide cyclase (AOC)* and *oxophytodienoate reductase (OPR)*. All these genes were significantly up-

regulated to various levels in both transgenic lines BG8 and BG9 and again showed even higher expression in mature leaf than the seedling leaf (Supplemental figure S4).

2.3 Targeted metabolite analysis of barley with the *Lr34* gene

To test for accumulation of the metabolites related to induced pathways, we first checked the total lignin content in three-week-old wild-type and transgenic seedlings. The transgenic plants accumulated around 20 percent more lignin than wild-type plants (Figure 2a). Even in the third leaf which was completely green at the time of sampling, there was more lignin in *Lr34res* barley suggesting that the process of lignin accumulation starts very early. Similarly, we found a three-fold increase in the amount of the amino acids tryptophan (Trp) and phenylalanine (Phe) both in seedling as well as mature leaves (Figure 2b). As phenylpropanoids originate from Phe, these findings suggest that there could be major changes also in phenylpropanoid products other than lignin. Specifically, expression of the agmatine coumaroyl-transferase gene was found up-regulated, and the biosynthetic products of this enzyme, hordatines, exhibit phytoalexin properties (Stoessl and Unwin, 1970). The putative pathway leading to hordatines is shown in Supplemental figure S5. The accumulation of hordatines was investigated with targeted ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS). There was no significant increase in the amount of hordatines in green third leaves of *Lr34res* barley seedlings compared to control plants, whereas there was a significant accumulation of hordatines in second leaves which already showed yellowing from the tip of the leaf (Supplemental figure S6).

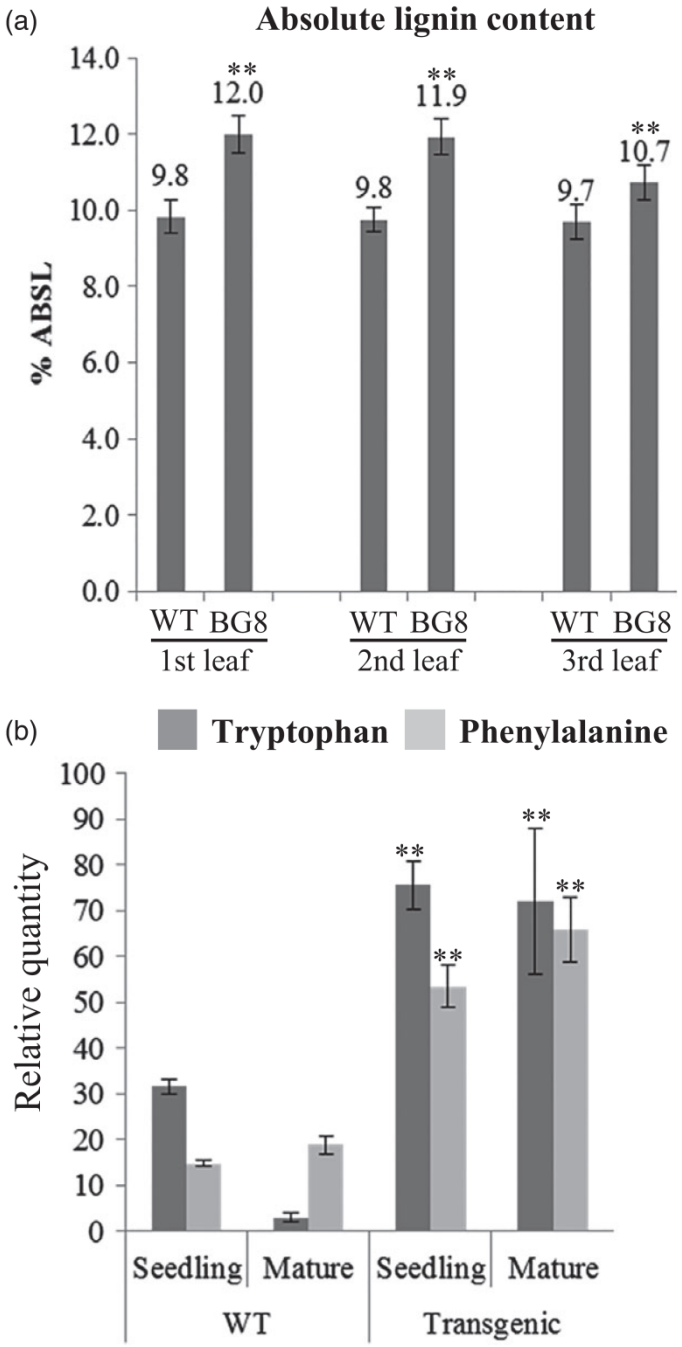

Figure 2: Measurements of total lignin and amino acids.

Measurements of total lignin (a) and amino acids (tryptophan and phenylalanine) (b). Total lignin content was measured in three-week-old seedlings of wild type and transgenic barley expressing *LR34res* in first, second and third leaf (ABCL=Acetyl Bromide Soluble Lignin). Tryptophan and phenylalanine measurements were done in seedling leaves and leaves from mature plants in both wild-type and *Lr34res* expressing transgenic plants of line BG8. Analysis was done in at least three biological replicates for lignin analysis and five biological replicates for amino acid. For tryptophan and phenylalanine (b) values on Y-axis represent relative quantity and error bars represent standard error (**=p value=<0.01, calculated by student t-test).

As described above several genes related to the JA-biosynthesis pathway were up-regulated in transgenic plants. It is known that *PR* genes are regulated by plant defense hormones such as jasmonic acid and salicylic acid (Mei et al., 2006; Wang and Dong, 2011). Therefore, we next measured the amount of these hormones in leaves of wild-type and transgenic plants. For this purpose, fully grown third leaves from three-week-old plants were sampled and analysis of hormones was done by UHPLC-MS/MS. As shown in Figure 3, *Lr34res* transgenic plants accumulated higher quantities of these defense hormones. There was a more than 10-fold increase of salicylic acid in these plants as compared to wild-type and also to transgenic plants with the *Lr34sus* allele. Similarly, *Lr34res* transgenic plants accumulated 3-5 fold more JA and JA-Ile than wild-type. Given these results in transgenic barley, we also checked the quantities of these hormones in transgenic wheat plants expressing *Lr34res* (Risk et al., 2012) and found that there was no significant difference in wild-type and transgenic plants under control conditions. However, there was a significant 4-fold increase in the amount of salicylic acid in transgenic wheat plants after rust infection (Supplemental figure S7).

Thus, the targeted metabolomics analysis confirmed the accumulation of various defense-related metabolites. This shift from normal metabolism towards a defense response might also explain the negative impact of *Lr34res* on barley plant vigour (Risk et al., 2013). Therefore, we tested two strategies to reduce the fitness costs caused by *Lr34res* in barley.

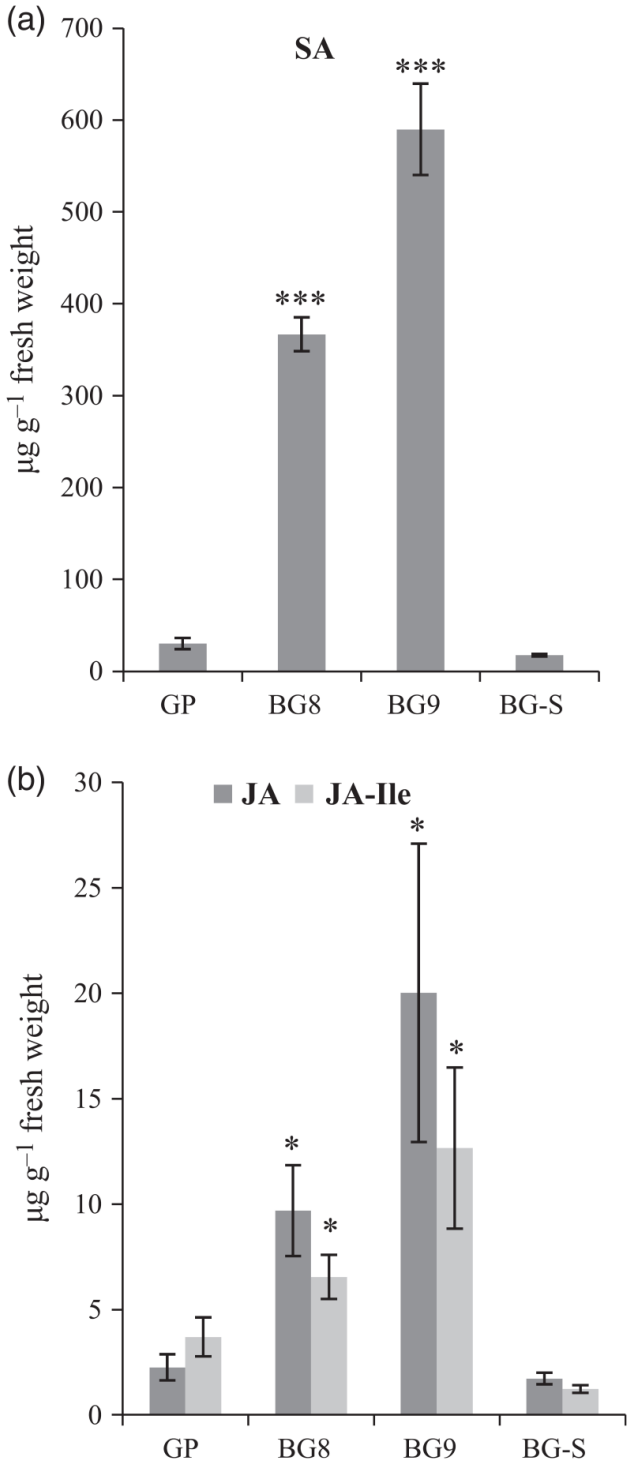

Figure 3: Accumulation of defense hormones in barley plants.

The measurements of salicylic acid (SA; a), jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile; b) were done in wild-type and transgenic barley plants expressing *Lr34res* (BG8 and BG9) and *Lr34sus* (BG-S). Hormone contents were determined in the third leaf of three-week-old seedlings. Values represent the mean of five biological replicates and the error bars represent standard errors (***=p value=<0.001, *= p value=<0.05 calculated by student t-test).

2.4 A single amino acid change is sufficient for Lr34-function

In wheat, *Lr34* exists in several allelic forms, however, so far only the *Lr34res* allele has been reported to provide disease resistance and LTN (Krattinger et al., 2009, Krattinger et al., 2011). There are two critical amino acid polymorphisms which differentiate Lr34res from all susceptible Lr34 proteins. In a first attempt to reduce the negative effects on barley growth we tested whether both amino acid changes are essential for *Lr*34res functionality in barley or if any of the two polymorphisms is sufficient. For this purpose, we generated two chimeric constructs resulting in two alleles encoding for the different amino acid polymorphisms, called *M1* and *M2* respectively. The M1 protein has a deletion of phenylalanine at position 546 (res) and a tyrosine residue at position 634 (sus), while the M2 protein has a phenylalanine at position 546 (sus) along with a histidine at position 634 (res) as shown in Figure 4a. Stable transgenic lines with these two chimeric constructs were developed in barley cultivar Golden Promise. 17 and 15 T0 transgenic plants were recovered for the *M1* and *M2* alleles, respectively. We observed that 15 out of the 17 transgenic T0 plants carrying *M1* developed the characteristic LTN phenotype associated with induced senescence and poor growth and development (Supplemental figure S8a). In contrast to *M1*, none of the T0 plants with the *M2* allele showed LTN. All 15 T0 plants grew similar to the wild-type and produced more seeds than *M1* plants. We further checked the expression of the *M1* and *M2* alleles in T1 progenies of three T0 plants each for *M1* and *M2*. Expression levels of *M1* were comparable to the *Lr34res*-containing line BG8. On the other hand, expression of the *M2* allele was very weak compared to *M1* or *Lr34res* in BG8 (Supplemental figure S8b). Similarly to these observations, only the resistance-conferring *Lr34res* was highly expressed in transgenic barley lines whereas *Lr34sus* expression levels were very low (Risk et al. 2013). We also analyzed the segregating T1 progeny plants of multiple lines of both the *M1* and *M2* alleles for rust resistance as well as for LTN and found that transgenic plants with the *M1* allele showed rust resistance as well as LTN (Figure 4b & 4c). The LTN phenotype was co-segregating with the rust resistance in a ratio of 3:1. On the other hand, all the transgenic events

with the *M2* allele were completely susceptible and showed no sign of LTN. This suggests that the deletion of the phenylalanine is required and sufficient to provide Lr34res-based disease resistance and LTN in barley. Based on these results, we also transformed wheat plants (cv. Bobwhite) with the *M1* allele. We found that transgenic wheat plants expressing the *M1* allele showed partial resistance to wheat leaf rust in a cold seedling leaf rust assay (Supplemental figure S9), confirming that the Phe deletion is sufficient for disease resistance also in wheat.

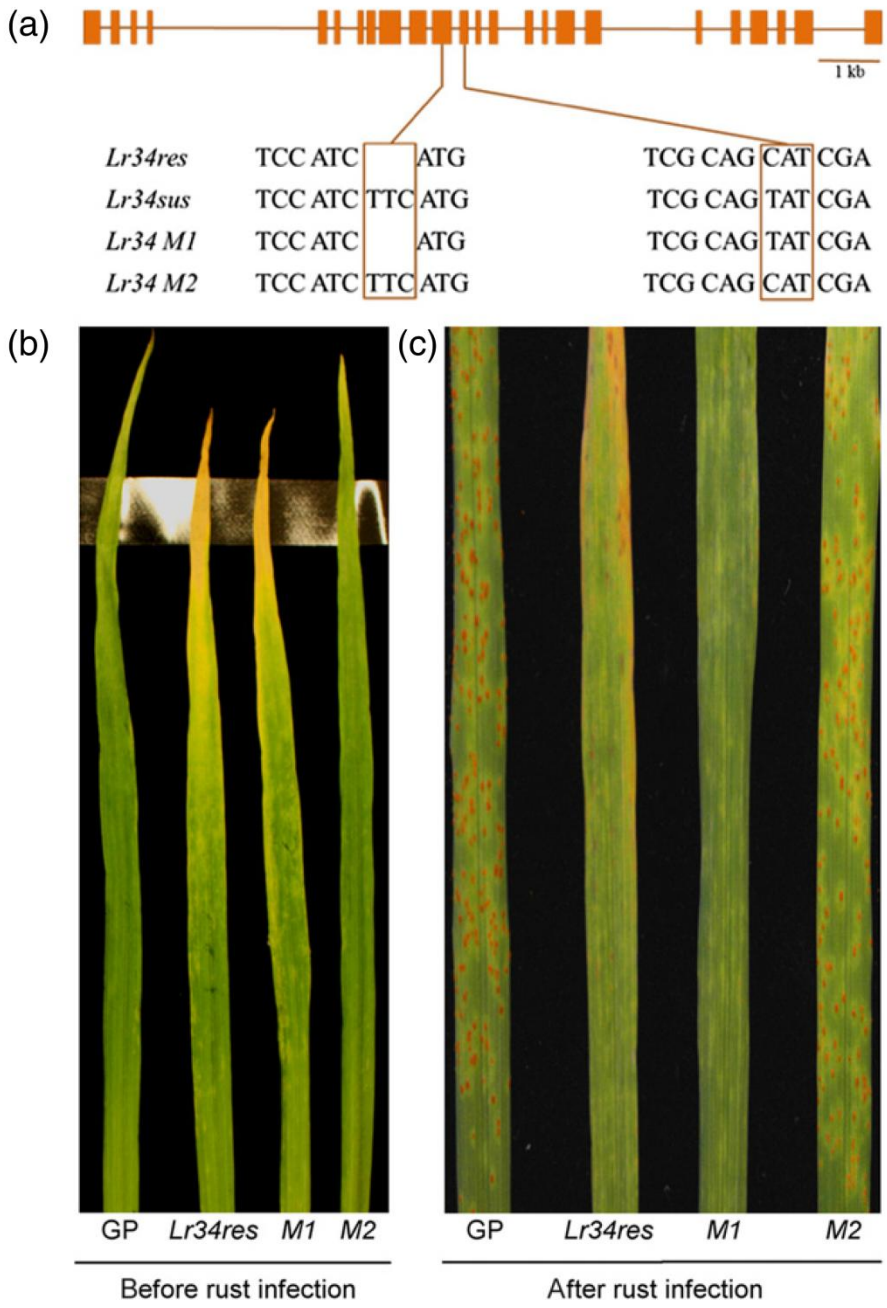

Figure 4: Generation of new *Lr34* alleles.

(a) Schematic representation of new *Lr34* alleles *M1* and *M2*, generated by site-directed mutagenesis and comparison of leaves from wild type and plants expressing *Lr34res*, *M1* and *M2* allele. The exons are indicated by boxes and introns are indicated by adjoining lines. The two critical codons for phenylalanine and histidine/tyrosine in exons 11 and 12 differentiating the *Lr34* alleles are shown in a box. Analysis of leaf tip necrosis phenotype (b) and rust resistance (c) in the third leaf of three-week-old barley seedlings of wild-type (GP: cultivar Golden Promise) and transgenic plants expressing *Lr34res* (BG8), *M1* and *M2* alleles, respectively. For rust-infected plants (c) at least six replicates were used and representative pictures were taken seven days after rust infection.

We also wanted to study if the metabolic consequences of the *M1* allele in barley are identical to those caused by the *Lr34res* allele. Therefore, we measured the amount of defense hormones in the third leaf of transgenic *M1* or *M2* barley seedlings. As expected, we found that plants with the *M1* allele accumulated higher amounts of these hormones in comparison to both wild-type and *M2* plants and similar to the levels of plants with *Lr34res* allele (Figure 5). We conclude from these experiments that a single amino acid deletion is sufficient for all observed changes mediated by Lr34res in barley.

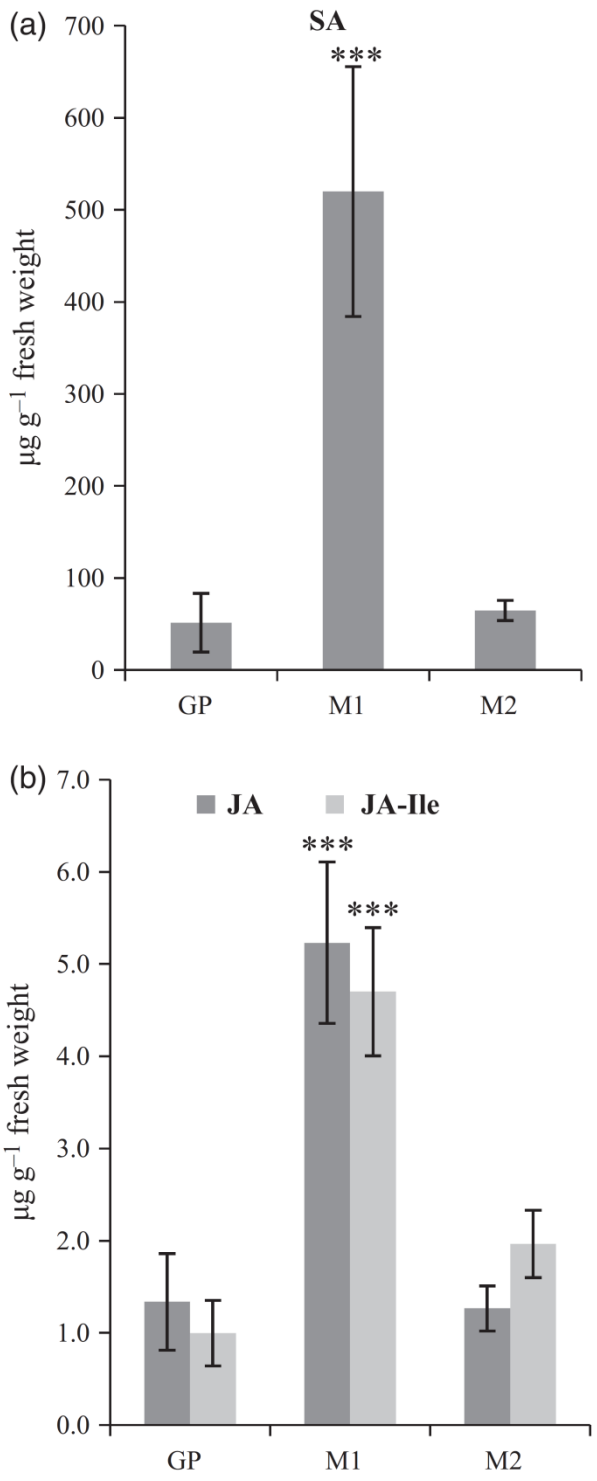

Figure 5: Accumulation of defense hormones in wild type and transgenic plants expressing *M1* or *M2* alleles.

Measurements of salicylic acid, SA (a) and jasmonic acid, JA (b) were done in third leaves of three-week-old barley seedlings of wild-type (GP: cultivar Golden Promise) and transgenic plants expressing *M1* and *M2* alleles, respectively. Values represent the mean of five biological replicates and error bars represent standard errors (***=p value=<0.001, calculated by student t-test).

2.5 The combination of the *Lr34res* and *Lr34sus* allele can attenuate negative growths effects without compromising resistance activity

In contrast to barley, the presence of *Lr34res* in hexaploid wheat is not associated with a noticeable impact in most environments. Wheat contains an *Lr34* homeolog of the susceptible haplotype on the B genome (Krattinger et al., 2011). We hypothesize that the presence of the susceptible *Lr34* haplotype might have a buffering effect that reduces the negative growth caused by *Lr34res*. While the *Lr34res* transcript levels were high in transgenic barley, *Lr34sus* showed very low transcript levels (Risk et al., 2013). To test if *Lr34sus* can have a buffering effect on *Lr34res*-mediated fitness costs, we crossed transgenic barley lines with *Lr34res* and *Lr34sus* alleles and analyzed the progeny plants in F_1 and F_2. For this, we used the two different *Lr34res* events BG8 and BG9 (Risk et al. 2013) and one *Lr34sus* event (SUS3). The combination of both alleles in F_1 and F_2 progeny plants of crosses with BG8 reduced the level of LTN in seedlings and in mature plants compared to progenies with only the *Lr34res* allele (Figure 6a and Supplemental figure S10). Transcript levels of *Lr34res* were ~8x – 20x reduced in F2 plants with both alleles compared to plants with only *Lr34res* (Supplemental figure S11). Interestingly, F_2 plants with both alleles showed rust resistance similar to lines with the *Lr34res* allele alone while wild-type and *Lr34sus* plants were susceptible (Figure 6b). Progeny of the BG9 crosses showed a different effect. In F2 plants containing both alleles, *Lr34res* transcript levels were not different from plants with only *Lr34res* (Supplemental figure S11). However, the transcript levels of *Lr34sus* increased ~13x – 16x when both alleles were present as compared to plants with only *Lr34sus.* Transcript levels of *Lr34res* are ~2.5x higher in BG9 compared to BG8 (Risk et al. 2013). These data suggest an interaction of *Lr34res* and *Lr34sus* on the transcript level. We hypothesize that the outcome of this interaction depends on the relative abundance of the two transcript variants and can either result in a down-regulation of *Lr34res* (cross with BG8 as a parent) or an up-regulation of *Lr34sus* (cross with BG9 as a parent).

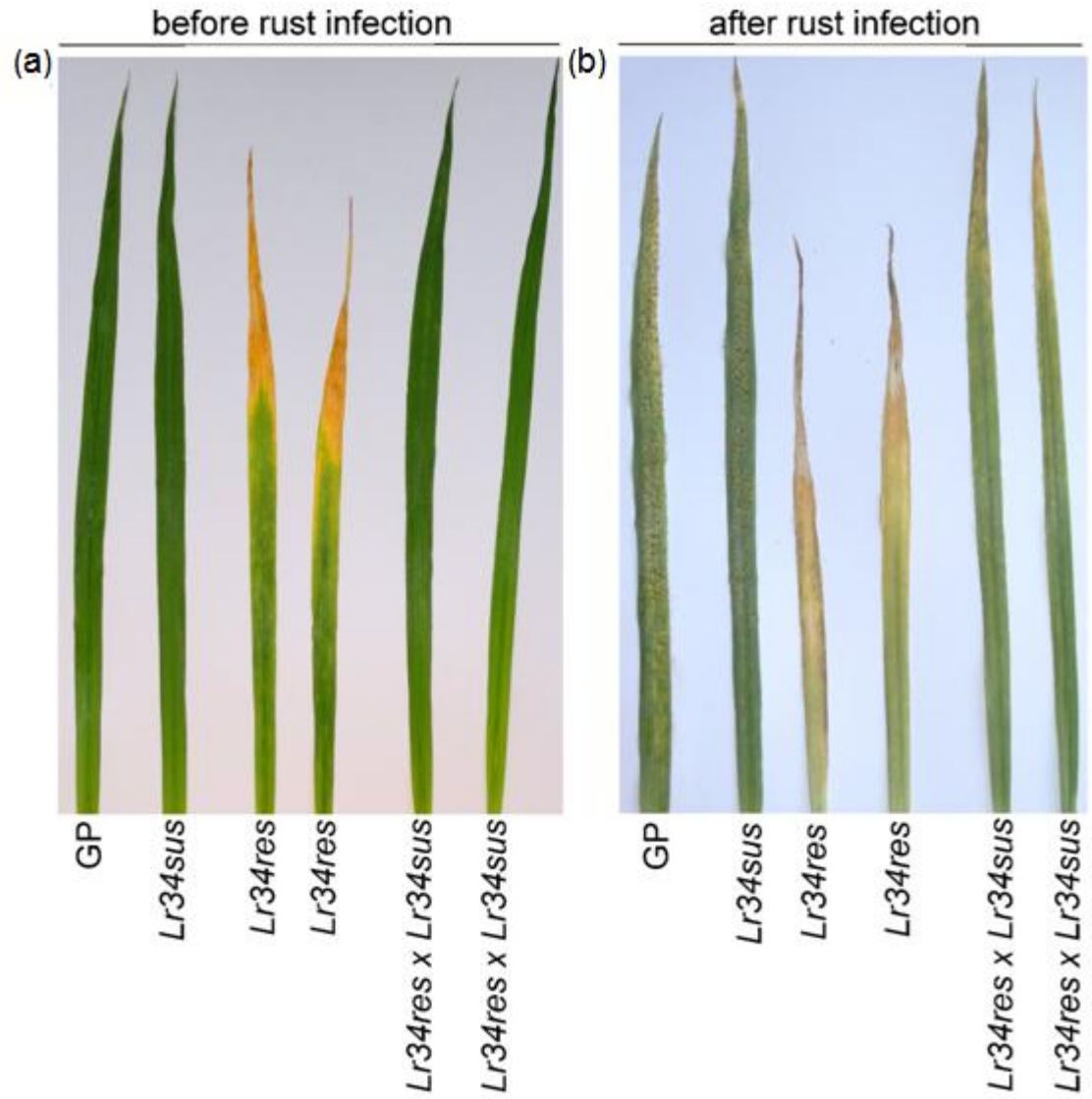

Figure 6: Effect of allele combination in BG8 crosses.

Analysis of leaf tip necrosis phenotype (a) and rust resistance (b) in the third leaf of three-week-old barley seedlings of wild-type (GP: cultivar Golden Promise) and transgenic plants expressing *Lr34res* (BG8), *Lr34sus* (SUS3) and F2 plants with both alleles (*Lr34res* x *Lr34sus*), respectively. For rust-infected plants (b) the photograph was taken seven days after rust infection.

As there was an increase in the amount of SA in plants expressing *Lr34res*, we next checked the hormone content in progeny of the BG8 crosses with both the *Lr34res* and *Lr34sus* alleles. Surprisingly, we found that plants with both alleles accumulated very similar levels of SA as in the wild-type or plants with the *Lr34sus* allele alone. However, since the plants with both alleles were significantly more resistant than both the wild-type and *Lr34sus* allele plants, we analyzed hormone levels after rust infection as well. After infection, plants with both alleles showed higher amounts of

SA than the plants without infection (Supplemental figure S12). We also checked basic yield parameters of the plants with both alleles of *Lr34* under greenhouse conditions. As shown in Supplemental figure S10, plants with both alleles were much greener and healthier and produced more biomass than plants with the *Lr34res* allele only. The plants with both alleles showed higher vigour throughout their life cycle and upon physiological maturity produced significantly higher above ground dry biomass, productive tillers and grains per plants as compared to the plants with *Lr34res* allele only and almost at par with plants with *Lr34sus* allele or wild-type plants (Supplemental table S3). Plants from allele combination and from WT or *Lr34sus* background were not significantly different. On the other hand plants with both alleles were significantly different from *Lr34res* (BG8). This observation in BG8 progeny reflects a wheat-like scenario where we have a natural combination of both *Lr34* haplotypes, the resistant haplotype on the D genome and the susceptible one on the B genome. Progeny of the BG9 crosses with both alleles showed phenotypes comparable to BG9, indicating that the down-regulation of *Lr34res* but not an up-regulation of *Lr34sus* reduces *Lr34res*-mediated fitness costs (Supplemental table S3). The attenuation of the negative effects in progeny of BG8 crosses is most likely not due to the zygotic state of *Lr34res* in segregating F2 progeny because both heterozygous and homozygous BG8 plants show severe LTN (Risk et al. 2013). Therefore, we conclude that the beneficial effects of *Lr34res* can be harnessed in transgenic barley by a combination of both alleles, with no or only minor effects on plant fitness.

3. Discussion

ABC transporters represent one of the largest gene families in plants, and Verrier et al., (2008) proposed 146 gene models in *Arabidopsis*. ABC transporters are involved in diverse processes related to pathogen response, lipid deposition, accumulation of phytate and transport of various phytohormones (Kang et al., 2011). The combined transcriptomic and targeted metabolomics approach revealed biochemical pathways which are modified in *Lr34res*-containing barley lines and are possibly involved in durable resistance against multiple fungal pathogens. Since *Lr34*-specific

changes occur in barley plants already at the very young seedling stage (along with rust resistance), we wanted to identify the transcriptomic changes caused specifically by expression of *Lr34res* in uninfected plants, without interference of gene expression changes caused by defense responses after rust infection. Many genes expressed in *Lr34res* barley might also be induced after rust infection in wild type and would therefore not be detected as genes induced by the presence of *Lr34res* if only infected plants would be studied. We hypothesize those changes seen in the two growth stages (seedling leaf vs. mature leaf) were due to the severity of LTN and not due to developmental stages.

3.1 Expression of *Lr34res* in barley causes transcriptional reprogramming resulting in constitutive defense responses

Lignin is an important plant metabolite and plays an important role in plant defense. Lignification of infected cells and formation of cell wall appositions represent important defense strategies against penetrating fungal pathogens such as powdery mildew (Zeyen et al., 2002). RNAi-mediated silencing of lignin biosynthetic genes resulted in susceptibility of wheat plants against powdery mildew fungus (Bhuiyan et al., 2009). We found that in transgenic barley expressing *Lr34res*, not only the biosynthetic genes but the lignin content also increased suggesting that *Lr34res* contributes to a constitutive, basal plant resistance in barley by increasing lignification.

Secondary metabolites often contribute to plant defense against pathogens. We have found several genes up-regulated which are potentially involved in the biosynthesis of secondary metabolites and phytoalexins such as *anthranilate synthase* (*AS*), *agmatine coumaroyltransferase* (*ACT*) and *flavonoid 7-O-methyltransferase* (*F7OMT*). Overexpression of barley *AS* has been shown to provide enhanced penetration resistance against barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (Hu et al., 2009). In barley, ACT catalyzes the synthesis of hydroxycinnamoylagmatines, which are immediate precursors of antifungal compounds known as hordatines. Although their exact function is not yet known, hordatines have been hypothesized to be involved in cell wall fortification and

cytotoxicity to invading pathogens (Burhenne et al., 2003). In the present study, we also found an up-regulation of the *ACT* gene as well as an increase in the amount of hordatines. Similarly, phytoalexins called avenanthramides were reported by Mayama et al., (1981) in oat (*Avena sativa* L.) infected with crown rust fungus (*Puccinia coronate* f.sp. *avenae*). Feeding experiments suggested that avenanthramides are *de novo* synthesized from anthranilic acid and L-phenylalanine (Ishihara et al., 1999). Christensen et al. (1998) found that in barley *F7OMT* is expressed after pathogen infection and that F7OMT is involved in the biosynthesis of the phytoalexin genkwanin. It is interesting to note that in the present study we found that all these genes are up-regulated in transgenic barley plants expressing *Lr34res*, even in the absence of any infection. These results suggest that *Lr34res* results in a constitutively active defense response.

In plants, phenylalanine and tryptophan are precursors for a large number of secondary metabolites such as alkaloids, flavonoids, phenylpropanoids, phenolic polymers, lignin and the plant growth regulator indole-3-acetic acid (Wasaka and Ishihara, 2009). Both Phe and Trp are synthesized from chorismate and the expression of their biosynthetic genes is affected by pathogen infection, elicitation and wounding (Niyogi and Fink 1992; Mobley et al., 1999). In the present study, we found that the amounts of Trp and Phe are much higher in transgenic barley in comparison to the wild-type in both seedling and mature leaves. This is also reflected by the fact that a number of downstream metabolites as well as associated gene expression are also altered.

PR proteins are small, secreted proteins with antimicrobial properties and there are 17 families of PR proteins described (Sels et al., 2008). We have observed that multiple *PR* genes were up-regulated in transgenic barley expressing *Lr34res*. Using tobacco plants it has been shown that constitutive expression of the *PR1* gene enhanced tolerance to pathogenic oomycete *Phytophthora parasitica* while silencing of *PR1* gene resulted in higher susceptibility (Alexander et al., 1993; Riviere et al., 2008). It has been proposed that PR proteins work together and are often encoded by multiple gene clusters (Silverstein et al., 2005).

SA has been suggested to induce systemic acquired resistance (SAR) as transgenic plants with reduced SA content are defective in SAR and exogenous application of SA can induce SAR (Gaffney et al., 1993; White, 1979; Wildermuth et al., 2001). Besides inducing SAR in plants, SA is also known to inhibit the hypersensitive response during effector-triggered immunity (Devdas and Raina, 2002). Both actions of SA, i.e. induction of SAR and inhibition of HR are in accordance with the action of *Lr34res* mediated resistance as we found that several SAR-associated genes are up-regulated in barley with *Lr34res* and there was no symptom of HR on the leaves with or without infection (Risk et al., 2012; 2013). It is interesting to note that in the present study we found that transgenic barley plants accumulated higher amounts of both SA and JA, suggesting, that *Lr34res* mediates a rather general defense response towards a broad range of pathogens, again supporting the nonspecific mechanism of resistance.

3.2 Action of *Lr34res* resistance in barley is based on a combination of constitutive defense responses

Hulbert et al. (2007) hypothesized that the broad-spectrum resistance and lack of race-specificity of *Lr34res* is reminiscent of SAR, involving induced expression of PR proteins prior to local infection by a pathogen. However, this was not confirmed by transcriptomic analysis of *Lr34res* action in wheat: typical PR proteins were up-regulated only after the rust infection and not in mock infection, which could be due to the presence of additional regulatory factors in wheat. There is a good correlation in the types of genes that are induced by *Lr34res* in wheat and barley. However, the time point of this induction markedly differed between wheat (Hulbert et al., 2007) and barley which might be due to wheat proteins additively interacting with Lr34res (Kolmer et al., 1996). Most genes that were induced in wheat only after rust infection were constitutively expressed in transgenic barley plants already before infection. Among these genes were most *PR* genes, *chitinases*, *wheat WIR1A* and *peroxidase* genes. The combination of several constitutive defense responses in barley with *Lr34res* easily explains the observed high resistance against rust and mildew pathogens already at the seedling stage.

3.3 *Lr34res*-mediated resistance and plant fitness

The fitness costs of defense responses are evident from *Arabidopsis* mutant plants constitutively expressing defense pathways that are stunted in their growth and development, while mutant plants defective in defense responses are taller (Heil and Baldwin, 2002), suggesting that up-regulation of defense related pathways is compensated by down-regulation of other metabolic processes such as photosynthesis and chlorophyll biosynthesis (reviewed by Rojas et al., 2014). This is consistent with our observation in transgenic barley where we found that genes and pathways related to photosynthesis and chlorophyll biosynthesis are down-regulated and pathways related to basal (such as lignin) and inducible defense responses (such as phytoalexins and PR proteins) are up-regulated. These constitutive, multiple defense mechanisms are expected to result in high energy demands in transgenic barley expressing *Lr34res*. Previously, Bolton et al. (2008) also proposed that broad-spectrum race non-specific resistance mediated by *Lr34res* in wheat is energy demanding and that this high energy demand cannot be maintained over longer periods of time. This could also explain why barley transgenic plants expressing *Lr34res* grow poorly and develop slowly, but show multiple pathogen resistance.

Several of the observed effects of *Lr34res* in barley could represent pleiotropic effects in the transgenic lines. However, by comparing expression patterns in very young seedling leaves that do not yet show growth or stunting defects with more adult leaves we can distinguish early effects from later effects which are more likely to be pleiotropic. Ultimately, the distinction of pleiotropic effects from the physiological effect directly caused by the *Lr34res* gene will only be possible once the substrate of the Lr34 transporter is known and immediate consequences of its transport can be determined.

3.4 Generation of new functional alleles of *Lr34*

For plant disease resistance breeding, durable resistance is the major goal, but there are only very few known sources for such resistance, among them *Lr34res.* The transfer of this gene to barley may provide a useful new source of resistance effective against multiple pathogens. To possibly expand molecular diversity of this resistance resource, we engineered a unique resistance allele (named as *M1*) of wheat *Lr34* through site-directed mutagenesis which was functionally active when tested in transgenic barley and wheat. Analysis of transgenic barley and wheat plants revealed that the *M1* allele provided disease resistance, but there was still enhanced LTN and poor growth in barley transgenic plants. Nevertheless, the observation that a single amino acid change results in the conversion of a susceptible to a resistant protein provides an opportunity to further test additional proteins of Lr34 by changing the phenylalanine residue to some other amino acid. Dakouri et al. (2010) analyzed 700 wheat genotypes and found that two of them showed an *Lr34* allele leading to a deletion of the same phenylalanine. These two lines were described as susceptible, with a disease severity of 10-80 percent. This is different from our findings which show that the deletion of phenylalanine is sufficient for resistance and LTN function. However, since *Lr34res* provides partial disease resistance in wheat, there is a need for additional analysis regarding disease severity. In addition, gene expression and full-length transcripts of *Lr34res* need to be verified in those two genotypes.

It has been shown that a change in a single amino acid can be critical for the substrate binding and transport function of ABC transporters. For example in the *Arabidopsis* AtPDR9 protein, a single amino acid change (alanine to threonine) increased the protein stability, thereby increasing resistance towards auxinic herbicides (Ito and Gray, 2006). In humans, a single amino acid mutation of glutamic acid to lysine in mitochondrial ABC transporter ABCB7 reduced cytosolic iron/sulphur protein function and caused X-linked sideroblastic anemia and cerebellar ataxia (Allikmates et al., 1999; Bekri et al., 2000). Thus, it is possible to genetically engineer and test new alleles of important

ABC transporter genes by manipulating critical residues and to harness a desirable trait in crop plants.

3.5 Attenuation of *Lr34res* induced LTN in barley by allele combination

Expression of *Lr34res* in barley induced premature LTN starting very early in the plant development and continued throughout the plant life, causing severe reduction in growth and development retardation. However, plants expressing *Lr34sus* showed no such detrimental effects (Risk et al., 2013). Previously, it has been found that *Lr34res* mediated disease resistance is affected by genetic background and possible involvement of additional genetic factors both in classical wheat breeding (Kolmer et al., 1996) and in transgenic wheat (Risk et al., 2012). Thus, in future studies it is important to study if one can see the similar effect in other barley genotypes or any other cereal crop, when using *Lr34res* for disease resistance. In wheat it is known that expression of *Lr34res* causes minimal losses in a disease-free environment as far as yield is concerned (Singh and Huerta Espino, 1997). In addition to *Lr34* (*res* or *sus*) on chromosome 7D in hexaploid bread wheat there is a homoeologous copy of *Lr34* residing on a translocated fragment of chromosome 7B (Krattinger *et al*., 2011). The two critical amino acids in this homoeologous copy are conserved with the Lr34sus protein. Recently, Stirnweis et al. (2014) described a suppression phenomenon in *Pm3*-based resistance during allele pyramiding in wheat. *Pm3* is an NB-LRR type resistance gene and they found reduction in *Pm3*-based powdery mildew resistance when different alleles of *Pm3* were stacked in transgenic wheat plants. This suppression occurred at the post-translational level as the levels of RNA and proteins were not changed (Stirnweis et al., 2014). We hypothesize that the Lr34sus protein though unable to provide disease resistance, possibly acts in fine tuning of the activity of Lr34res. Our data suggest that this process occurs at a transcriptional level rather than post-transcriptional. Breeding for inbuilt disease resistance is the most promising way for environmentally sustainable agriculture. In the present investigation we characterized the mechanism of one of the most abundantly used

resistance gene in wheat breeding and found a strategy to use *Lr34res* in heterologous systems which might be successfully extended to other crop species.

4. Experimental procedures

4.1 Cloning of new *Lr34* alleles and generation of stable transgenic *Lr34* barley lines; growth and infection analysis

Cloning of *Lr34res* and *Lr34sus* alleles and the construction of binary vectors were described previously (Risk et al., 2013). In brief, cloning was done in binary vector p6U and entire coding regions of *Lr34res* and *Lr34sus* alleles were cloned including 2.35 kb native promoter and 1.75 kb native wheat terminators. For the generation of new alleles (*M1* and *M2* alleles), we used the *Lr34sus* allele construct and employed a PCR based, site-directed mutagenesis protocol provided in the Quick change site directed mutagenesis kit (Agilent Technologies, CA USA) with the help of primers M1-Fwd (5' GCATTATTTTTTTCCATCATGATTATG 3') / M1-Rev (5' CATAATCATGATGGAAAAAAATAATGC 3') for the *M1* allele and M2-Fwd (5' CAGTCACCTCGCAGCATCGATTTATTG 3') / M2-Rev (5' CAATAAATCGATGCTGCGAGGTGACTG 3') for the *M2* allele respectively. Confirmation of desired mutations and vector integrity was made by sequencing the entire binary constructs prior to barley transformation. *Agrobacterium*-mediated stable transformation of barley cv. Golden Promise was done as described previously (Hensel et al., 2009; Risk et al., 2013). Stable transgenic plants were selected for both new constructs and expression of the transgene was confirmed as described earlier. For pathogen infection tests and other plant growth parameters transgenic plants were grown in standard glasshouse conditions and rust infections were carried out as described in the same study (Risk et al., 2013). Transformation of bread wheat cv. Bobwhite with the *M1* allele was carried out as previously described (Risk et al., 2012).

4.2 Microarray hybridization and data analysis

The details of barley microarray chip used, hybridization and data analysis is given in Supplemental methods. The microarray data is submitted in NCBI GEO repository with study accession number GSE64228. For annotation of differentially expressed probe sets BLASTX analysis was done using the annotated rice genome as a reference (Supplemental table S1 and S2).

4.3 Gene Ontology and biochemical pathway analysis

GO annotation analysis on gene clusters was performed using the BiNGO 2.3 plugin tool in Cytoscape version 2.6 with GO_full and GO_slim categories, as described by Maere et al. (2005). First, best homeologous rice genes were identified by BLASTX with a cut off e-value of 10E-10 using the nucleotide sequences of barley probe sets and then those rice locus identifiers were used as input for BiNGO analysis, both in seedling and mature leaves. Over-represented GO_full categories were identified with a significance threshold of 0.05 after a Benjamini and Hochberg FDR correction (Benjamini and Hochberg, 1995). For the identification of biochemical-metabolic pathways related to differentially regulated genes we used MapMan omics analysis tool (Usadel et al., 2009) with default settings and barley mappings.

4.4 Quantitative PCR-based expression analysis

Quantitative transcript measurements in wild-type and transgenic barley lines containing the genomic *Lr34res* transgene were carried out on RNA isolated from third leaves without LTN from 14-day-old plants or the mature leaf with LTN from ten-week-old plants with three biological replicates. Primers for the target genes were designed by using Primer Express 3 software (Life technologies, CA USA) and sequences are listed in Supplemental table S5. qPCR experiments were carried out as previously described and for qPCR of various *Lr34* alleles, primers described in Risk et al., (2013) were used.

4.5 Measurement of lignin and hordatines content

Lignin estimation was performed according to Fukushima and Hatfield (2005) using the acetylbromide soluble lignin (ABSL) method. Hordatine levels were measured as described by (De Vos et al., 2007). The detailed procedures followed are given in Supplemental methods.

4.6 Measurements of hormones and amino acids

Hormone contents were measured in leaf tissues of wild-type and transgenic barley by ultrahigh pressure liquid chromatography- tandem mass spectrometry (UHPLC-MS/MS) analysis as described by Glauser et al. (2014). Phenylalanine and tryptophan were quantified relatively by UHPLC-MS according to Marti et al. (2013). The measurements were done in at least five biological replicates, each representing five different plants.

4.7 *Lr34res* and *Lr34sus* allele combination by crossing transgenic plants

After a careful segregation analysis at T2 generation BG8/33 transgenic progeny line was selected for crossing (Supplemental table S6). To generate plants with both alleles of *Lr34*, crossing was done in greenhouse-grown, segregating T2 transgenic plants with *Lr34res* and *Lr34sus* allele respectively. Both transgenic lines with the *Lr34res* allele (BG8 and BG9) were used as parents, as well as transgenic line BGS3 (SUS3) with the *Lr34sus* allele (Risk et al., 2013). The progeny F_1 plants were selected for the presence of both alleles by molecular markers described in Lagudah et al., (2009). qPCR expression analysis of individual alleles was done in the third leaf of three-weeks-old plants of the F_2 generation, as described earlier (Risk et al., 2013), except that the additional reference gene *GAPDH* with the primers sqRTF-HvGAPDH 5' CCGGGTTCCCACTGTGGAT 3' and sqRTR-HvGAPDH 5' TGACTAGCAACTCGGTGCGG 3' was also used here. For the determination of yield parameters, F_2 progeny plants along with wild-type and control plants with either the *Lr34res* or *Lr34sus* allele were grown in the greenhouse and parameters such as total above ground biomass, productive tiller number, and grain weight per plant were recorded at physiological maturity.

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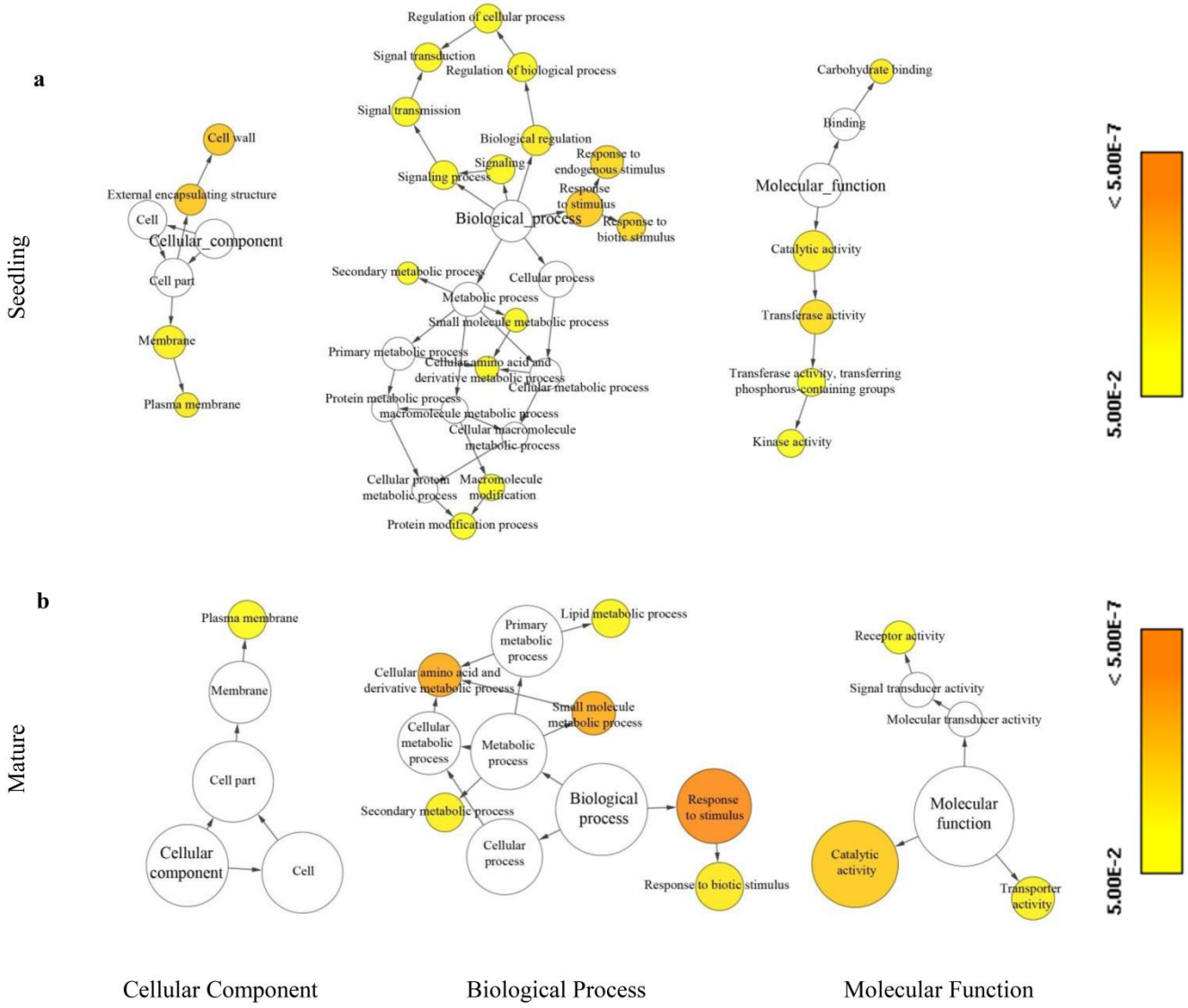
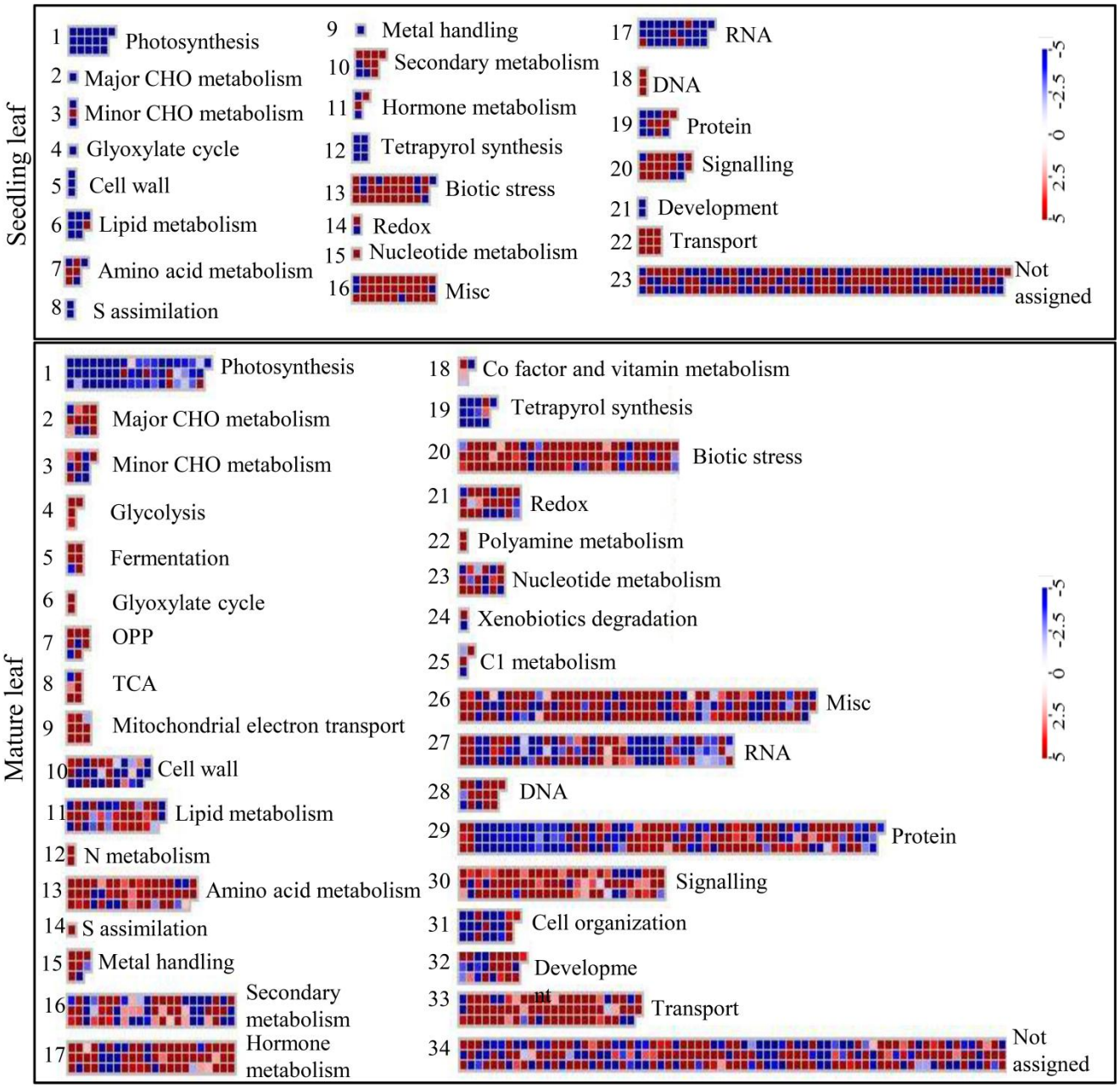
7. Supplemental material

7.1 Supplemental figures and Supplemental tables



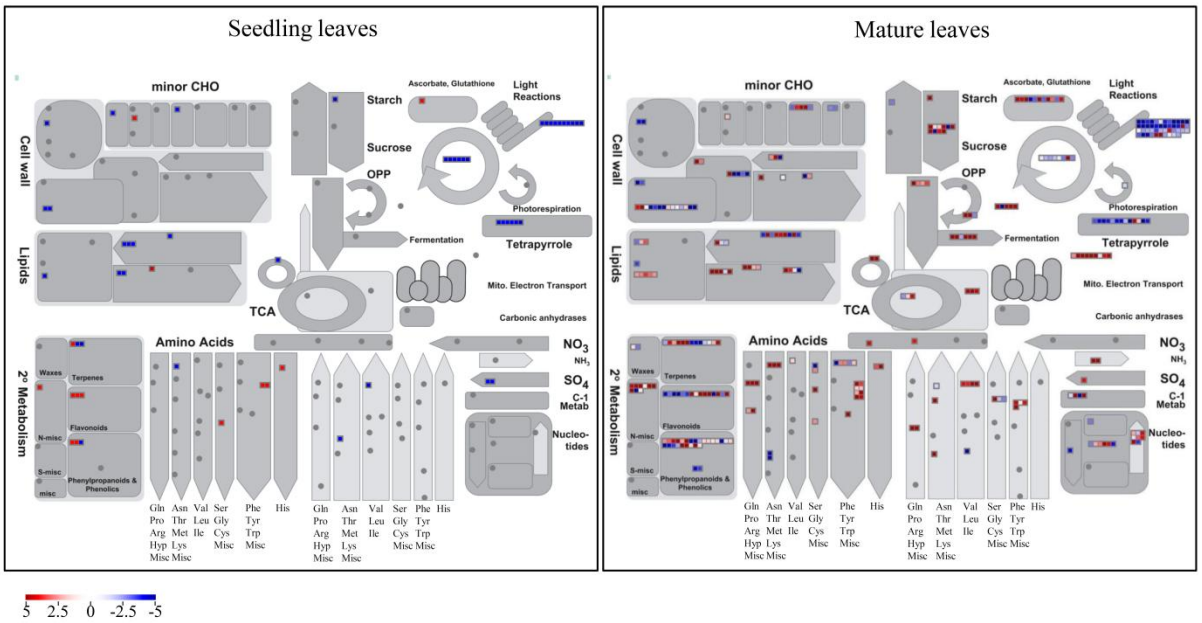
<u>Supplemental figure S1: Enriched gene ontology (GO) terms for up-regulated genes in transgenic barley lines expressing the wheat *Lr34res*.</u>

(a) Seedling stage, (b) Mature leaf stage. The node size reflects the number of genes attributed to the respective category. Colored nodes represent GO terms which are significantly overrepresented (corrected P value of <0.05). More intense color indicates higher statistical significance as indicated on the right of the Figure.

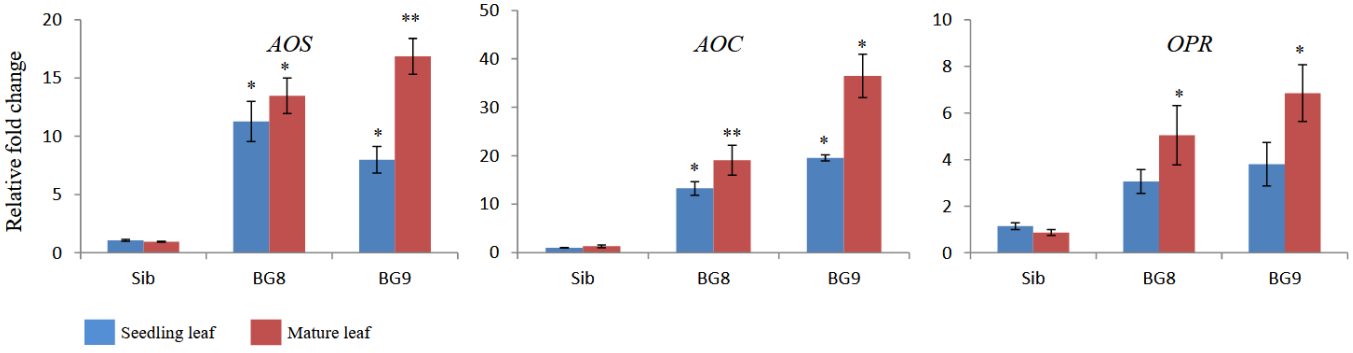


Supplemental figure S2: MapMan maps for overview of transcriptomics.

Maps showing differences in transcripts belonging to different functional classes between wild-type (Sib) and transgenic barley in two growth stages, seedling and mature leaves. In the color scale, blue represents genes down-regulated by at least 5-fold in transgenics and red represents 5-fold up-regulation in seedling and mature leaf stages.

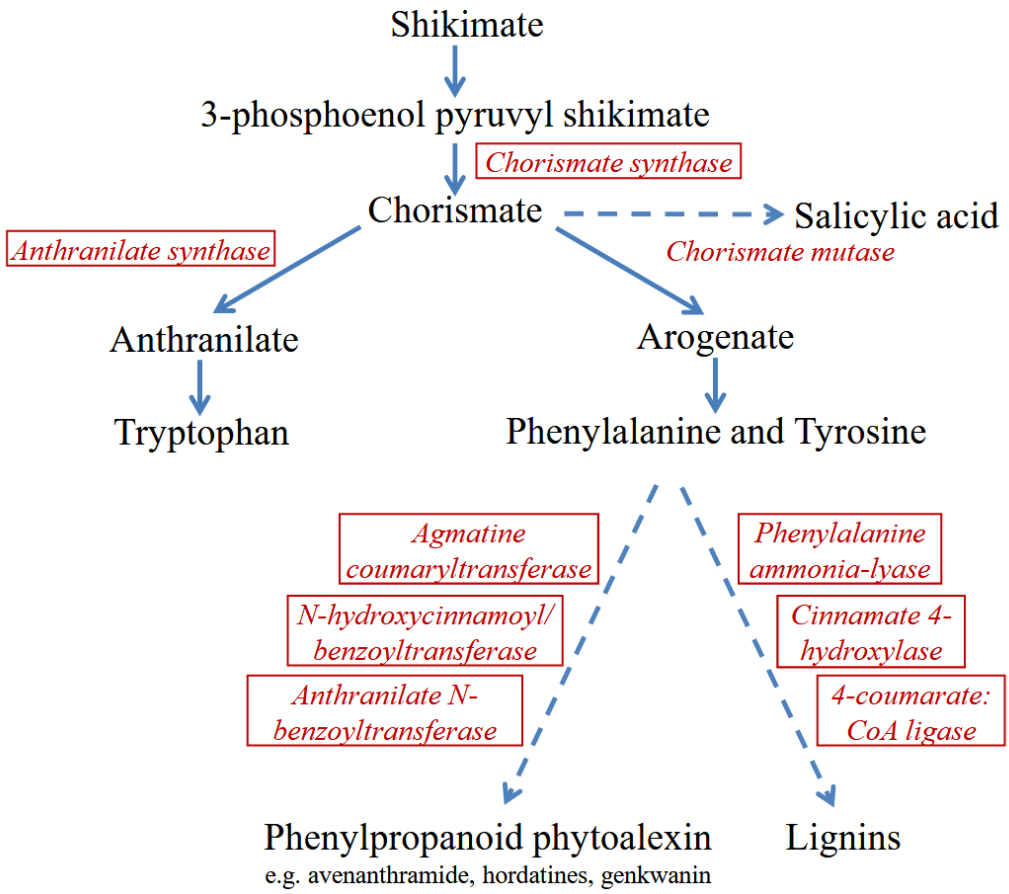


Supplemental figure S3: MapMan maps for metabolism overview.
Maps showing differences in transcript levels between wild type (Sib) and transgenic barley expressing *Lr34res* in two growth stages, seedling (left) and mature (right) leaves. In the color scale, blue represents genes down regulated by at least 5-fold and red represents 5-fold up-regulation in seedling and mature leaf stage.



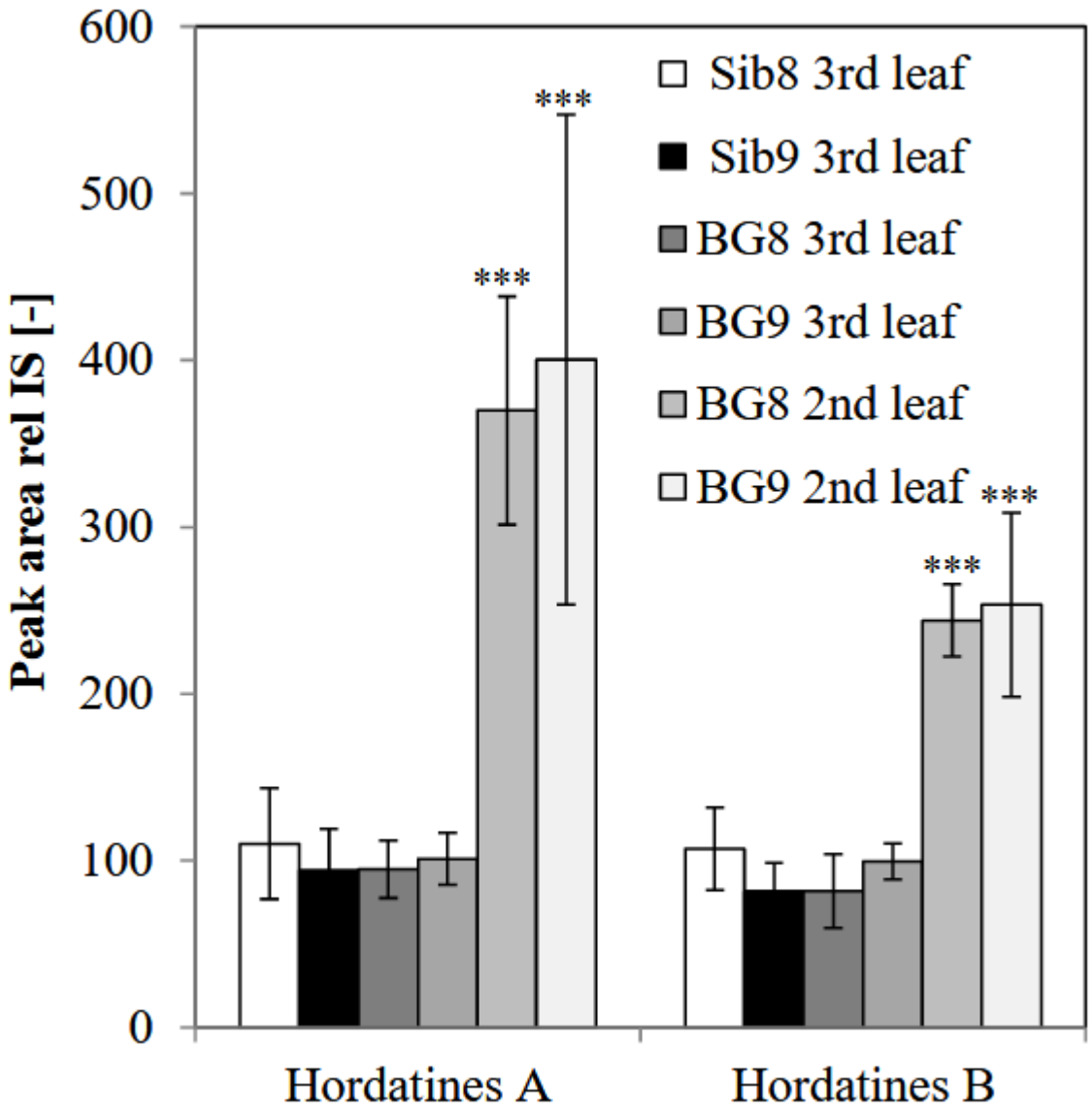
Supplemental figure S4: qRT-PCR expression analysis of genes involved in the biosynthesis of JA in wild-type (Sib) and transgenic barley.

qPCR analysis were performed in wild-type (Sib) plants and plants from two transgenic lines expressing *Lr34res* (BG8 and BG9) and two growth stages, seedling leaves and mature leaves. At least three biological replicates and three technical replicated were used for analysis and values on Y-axis represent relative transcript abundance compared to Sib and error bars represent standard error. (AOS=allene oxide synthase, AOC= allene oxide cyclase, OPR=oxophytodienoate reductase) (*=p-value=<0.05; **=p-value=<0.01, calculated by student t-test).



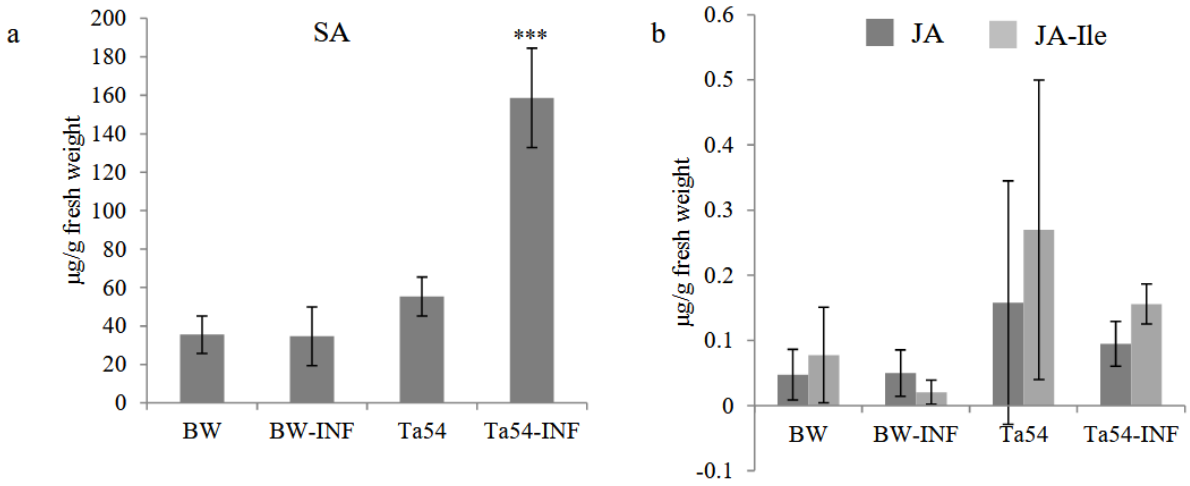
Supplemental figure S5: Hypothetical representation of *Lr34res*-mediated biochemical pathways in transgenic barley plants.

The enzyme names are given in red color and the red box indicates the genes which are up-regulated in transgenic barley plants expressing *Lr34res*. (modified from Caldo et al., 2004).



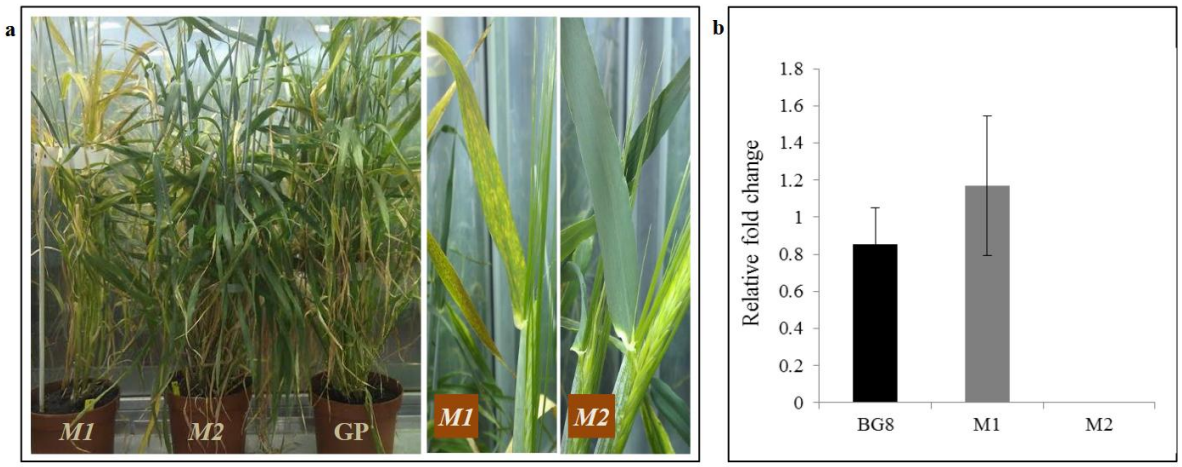
Supplemental figure S6: Quantification of hordatines.

Relative quantification of hordatines in barley seedlings based on extracted ion chromatograms of the [M+2H]2+ ions. Sib = sister line, BG8, BG9 = transgenic lines, peak areas are given relative to internal standard ampicillin, values are means of five biological replicates and error bars represents standard error (***=p-value=<0.001, calculated by student t-test).



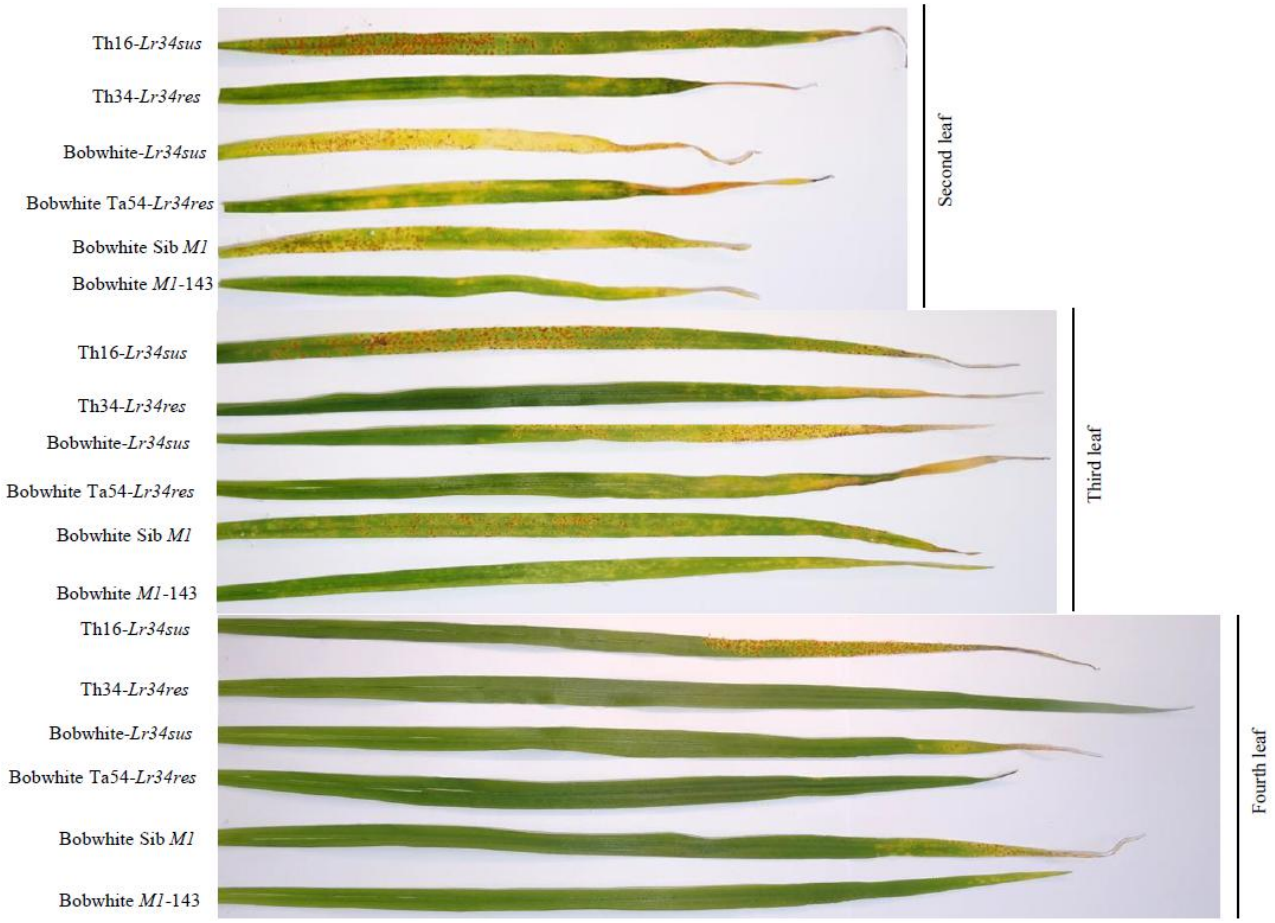
Supplemental figure S7: Accumulation of defense hormones.

Accumulation of defense hormones, salicylic acid (SA) and jasmonic acid (JA) in wild-type cv. Bobwhite (BW) and transgenic wheat plants of line Ta54 expressing *Lr34res*, before and after rust infection (-INF). At least five biological replicates were used for analysis and error bar represent standard error (***=p-value=<0.001, calculated by student t-test).



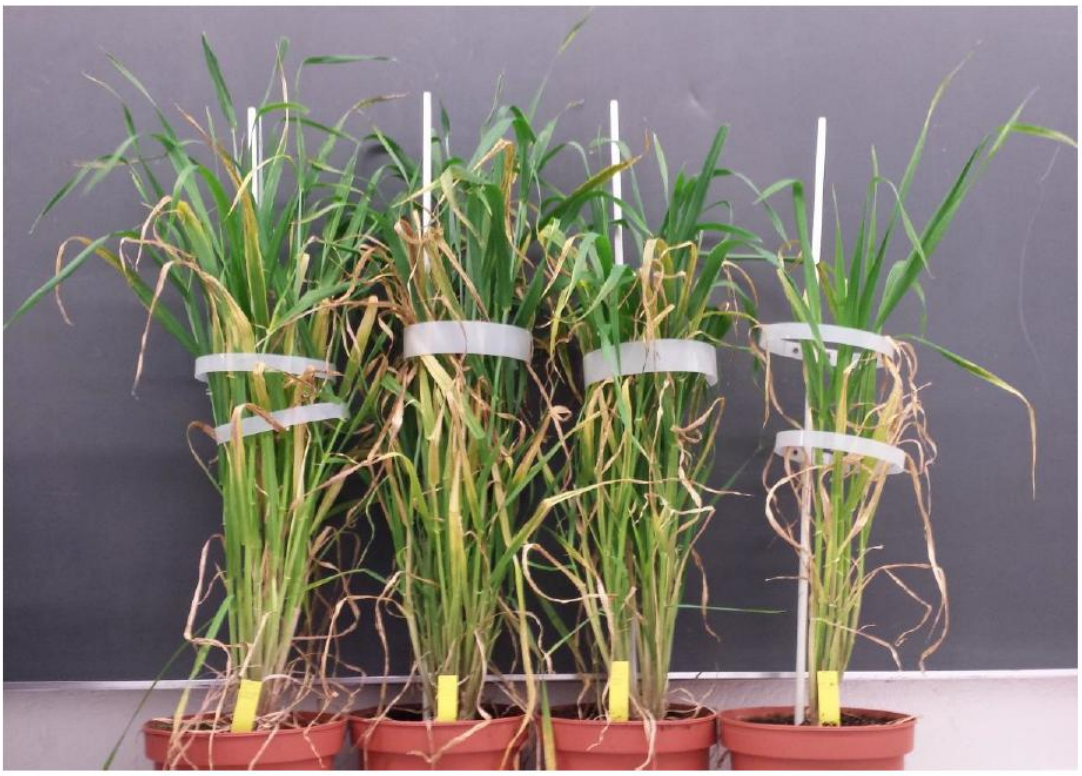
Supplemental figure S8: Expression of new *Lr34* alleles (*M1* and *M2*) in barley.

(a) Heterologous transcript levels of new *Lr34* alleles (*M1* and *M2*) in barley showed induced LTN by the *M1* allele. (b) RT-qPCR analysis of the *Lr34res* (BG8), *M1* and *M2* alleles. The values represent the mean expression of three different T1 transgenic families, respectively and error bar represent standard error. The transcript levels were normalized to the reference gene *GAPDH* and three biological replicates and three technical replicates were used for expression analysis. M1 = deletion of phenylalanine 546 with tyrosine 634; M2 = phenylalanine 546 with histidine 634.



Supplemental figure S9: Response of different wheat genotypes towards leaf rust.

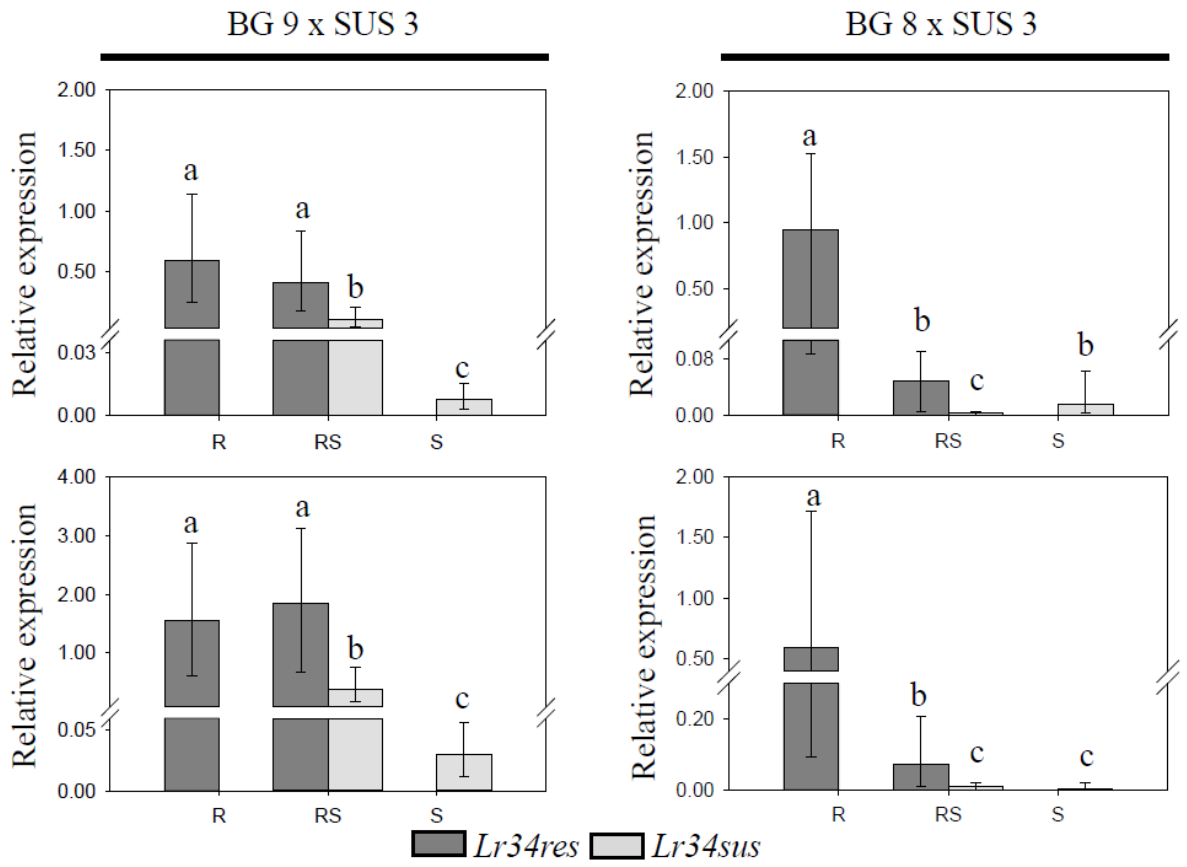
Two-week-old seedlings were infected with wheat leaf rust, pathotype Mackeller and grown for four weeks after infection at 4° C. Three different leaves (second, third and fourth leaf) are shown in the figure of genotypes viz. Thatcher isolines with *Lr34res* (Th34), *Lr34sus* (Th16), Bobwhite with *Lr34sus*, transgenic Bobwhite expressing *Lr34res* (Ta54), sib line of *M1* and transgenic Bobwhite expressing *M1* allele (*M1-143*).



GP *Lr34sus* *Lr34res* x *Lr34sus* *Lr34res*

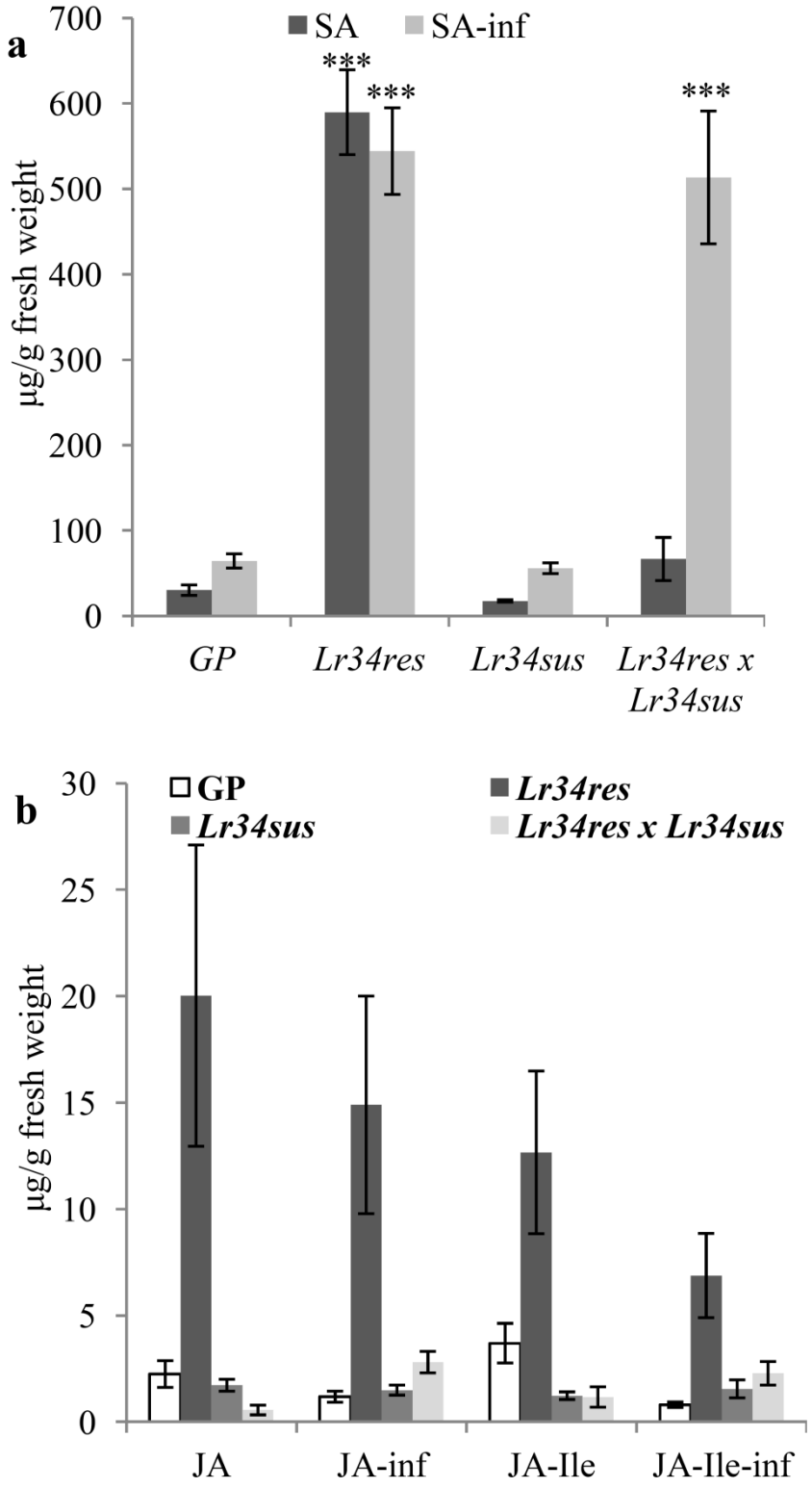
Supplemental figure S10: Effect of allele combination in transgenic barley having both *Lr34res* and *Lr34sus* alleles.

Eight-week-old mature plants grown in the greenhouse: wild-type plant from cultivar Golden Promise (GP), an F2 plant containing both *Lr34res* and *Lr34sus* alleles derived from a cross of BG8xSUS3. Transgenic barley having both *Lr34res* and *Lr34sus* alleles showed reduction of the LTN phenotype.



Supplemental figure S11: Relative transcript levels of *Lr34res* and *Lr34sus* alleles in F2 progenies of BG8 x SUS3 and BG9 x SUS3.

Two independent crosses were made and analyzed for each parental combination (upper and lower panel). Transcript levels were calculated based on ΔCq values using primers for *Lr34res*, *Lr34sus* and the reference gene *GAPDH*. Transcript levels for both alleles are shown in the same graph. Bars represent mean expression values of 3-4 biological replicates. Letters indicate lines with equivalent expression levels (Students t-test on Log10-transformed expression values, α>0.05). Error bars represent the 95% confidence intervals (back transformed). 'R' = F2 progenies with only *Lr34res*; 'RS' = F2 progenies with both alleles and 'S' = F2 progenies with only *Lr34sus*. The 3-4 biological replicates for 'R', 'RS' and 'S' represent a mixture of homozygous and heterozygous F2 progeny.



Supplemental figure S12: Accumulation of defense hormones in different transgenic barley.

Accumulation of defense hormones before and after infection (inf): salicylic acid (a) and jasmonic acid (b) in third leaves of three-week-old barley seedlings of wild type (GP: cultivar Golden Promise) and transgenic plants expressing *Lr34res*, *Lr34sus* and F2 plants expressing both alleles, respectively. Values represent the mean of five biological replicates and error bars represent standard error. For leaf-rust infected plants, hormone measurements were done seven days after rust infection (***=p-value=<0.001, calculated by student t-test).

Supplemental table S1: Differential expression in seedling stage.

This table consists of 14 pages. Therefore, it is available online:
http://onlinelibrary.wiley.com/store/10.1111/tpj.13001/asset/supinfo/tpj13001-sup-0002-TableS1.pdf?v=1&s=3d715afefe5d4aed77d5e9fff671a5151e3e9917

Supplemental table S2: Differential expression in mature leaf.

This table consists of 80 pages. Therefore, it is available online:
http://onlinelibrary.wiley.com/store/10.1111/tpj.13001/asset/supinfo/tpj13001-sup-0003-TableS2.pdf?v=1&s=c0cd68a5ad7b71d67266c9a7aa4da21f0e6d7dc6

Supplemental table S3: Agronomic performance.

Measurement of yield parameters in F2 progenies of crosses BG8 x SUS3 and BG9 x SUS3. Means and standard deviation are shown for dry biomass, number of tillers and seed weight per plant. * indicates significant difference from *Lr34res* with P<0.05 and ** P<0.01 (T-Test). BG8 and BG9 crosses were grown at different time points.

Allele combination	Number of plants analyzed	Dry biomass at maturity (g)	Number of productive tillers	Seed weight per plant (g)
BG8 x SUS3				
Lr34res / Lr34sus	12	50.9 (±14.4)*	22.7 (±10.6)**	20.1 (±8.7)**
Lr34res	6	19.7 (±4.0)	7.8 (±3.0)	3.8 (±1.7)
Lr34sus	6	53.4 (±8.2)*	23.7 (±5.0)**	24.2 (±6.7)**
WT	6	60.3 (±6.7)*	25.7 (±7.5)**	22.2 (±7.7)**
BG9 x SUS3				
Lr34res / Lr34sus	25	n.d.	17.4 (±8.3)	4 (±2.2)
Lr34res	9	n.d.-	17.8 (±10.8)	3.2 (±3.8)
Lr34sus	9	n.d.-	31.7 (±13.2)*	20.6 (±11.4)**

n.d.: not determined

Supplemental table S4: Primers for qRT-PCR.

Target Gene	Accession Number	Primer	Primer sequence (5'-3')
Lr34res	FJ436983	Fwd	GGGAGCATTATTTTTTTCCATCATG
		Rev	ACTGGCAGAAGAACCTTGAAACA
Lr34sus	FJ436985.1	Fwd	GGGAGCATTATTTTTTTCCATCTTCAT
		Rev	ACTGGCAGAAGAACCTTGAAACA
RLILP	AK359255.1	Fwd	TCTGTCTGGTGGCGAGCTT
		Rev	CCGCACTTTGTACAGCAACAAC
Phenylalanine ammonia lyase (PAL)	AK361880.1	Fwd	CTGCTAGAGGTGCAGTGGAGAA
		Rev	GGTATGACCGGCAGTCTACGAT
Cinnamate-4-hydroxylate	AK363917.1	Fwd	GCGCCATTGATCACATCCT
		Rev	GACCACAGCGTCGTCTCGAT
4-coumarate-CoA ligase	AK369473.1	Fwd	CATCACGAGAACGAGAACGA
		Rev	GTCTCCACGAACCGGTACAC
Cinnamyl alcohol dehydrogenase	AK353779.1	Fwd	CCTCCCCGAGAATCCTATGG
		Rev	CCGGCCAGGGTCTTGTT
Allene oxide cyclase	AK365667.1	Fwd	AGGTCAAGCTCAACCAGATCGT
		Rev	CGGGATGCCCTTGAGGTA
Allene oxide synthase	AK366287.1	Fwd	GGAGGCGGTGCACAACAT
		Rev	GACGGGAACAGGATCTTCATG
Oxophytodienoate reductase	KC661078.1	Fwd	CGACAGGGATGATGGAAATAAAG
		Rev	GCGCCCATATGCTACCAAGT
Flavonoid 7-O-methyl transferse	X77467.1	Fwd	CACCACCGCCAGAACCAT
		Rev	TCCAACACGGAGCACTTGAC
Anthranilate N-benzoyltransferase	AK356302.1	Fwd	GTTCCATCAGCTCGATTTTGGT
		Rev	GATCATGAGCCCCTCGAAAG
Anthranilate synthase	EU828766.1	Fwd	GAGGTGGCCCGGATCAA
		Rev	GCCGGAGTTGGCGAAGT
Agmatine coumaroyl transferase	AB334132.1	Fwd	TGACATGCTTATCGCTCTTGCT
		Rev	GTATTGTGGCTTGGAGCTGTTG

Xa21	AK367058.1	Fwd	TGATCTCAAGCCCAGCAATATTC
		Rev	GCCGAAATCCCCCAAATG
Pr1a	AK251770.1	Fwd	CGGTCGTGATAAGATTGTGAAACT
		Rev	ATCCATTAGCCGGTCTAAACCA
Pr1b	X74940.1	Fwd	CTGGAGCACGAAGCTGCAG
		Rev	CGAGTGCTGGAGCTTGCAGT
Pr2	AY612193.1	Fwd	TGTTCGCCATGTTCAACGA
		Rev	CCAAAGTGCTTCTCCGTGTCA
Pr3	AK368862.1	Fwd	GAGCTTGTGATTCGTGTATGTGTGT
		Rev	CGCTCACGCCATTAGCTCAT
Pr4	Y10813.1	Fwd	GCAATGGCTCACCTCCTTAGG
		Rev	GAGCGAGGCCAGCATCA
Pr5	AK355059.1	Fwd	CGCCGACCAACTATTCGAA
		Rev	GTCGTCCTTGGCATAGCTATAGG
Pr8	AL450587.1	Fwd	ACGGGCGCCAGCAACTA
		Rev	GCTGTTCTGCACGTCGTTGT
Pr9	AK354347.1	Fwd	GCTACGCTGTCTGAAGTCTGAACTAG
		Rev	CATTTCGTGTACACATGCACCTT
Pr10	AK361884.1	Fwd	CTGCCGAGTACGCCTAATTAAAC
		Rev	TTTCGAACAAAGGGAAAGTTGATT

Supplemental table S5: Segregation analysis of transgenic barley line BG8 expressing wheat *Lr34res*.

Event	Generation	Seeds analyzed	Seeds germinated or survived till seed production	Plants with LTN	Plants without LTN
BG8-4	T2	12	4	1	3
BG8-6	T2	12	4	0	4
BG8-12	T2	12	12	9	3
BG8-17	T2	12	10	8	2
BG8-31	T2	12	12	9	3
BG8-33	T2	12	12	9	3
BG8-42	T2	12	12	9	3
BG8-44	T2	12	6	4	2
BG8-51	T2	12	5	5	0

7.2 Supplemental methods

7.2.1 Microarray hybridization and data analysis

We used the Genechip^{R} barley genome array from Affymetrix to identify transcriptional changes in *Lr34res*-containing transgenic barley lines. The affymatrix barley array consists of 22,500 probe sets, derived from 400,000 ESTs which together represent 53,030 unigenes. For microarray analysis, 2 µg high-quality total RNA isolated with SV total RNA isolation kit (Promega, WI, USA) including on column DNAse digestion was taken as the starting material. RNA was isolated from the third leaf of three-weeks-old seedlings and mature leaf of eight-week-old plants of wild-type and transgenic lines (BG8 and BG9, Risk et al., 2013) grown under normal growth conditions. The analysis was performed at the Functional Genomics Centre of the University of Zurich (http://www.fgcz.ch) and the Affymetrix (Santa Clara USA) GeneChip Barley Genome Array was used for microarray hybridizations. Three independent biological replicates of all the samples were taken for the analysis. For the data analysis, the image (.cel) files were imported into Genespring GX 10.0 software (Agilent Technologies, CA, USA). The normalization of data was performed by the Gene Chip Robust Multiarray Analysis (GCRMA) algorithm. Differential expression analysis was performed on the log transformed data using the Student's *t*-test with microarray data obtained for mRNA derived from leaves of wild-type plants as reference tissue. The probe sets that were up- or down-regulated ≥4-absolute fold (log fold 2) with a *P* value <0.05 (with Benjamini Hochberg correction) in transgenic as compared to wild-type were identified.

7.2.2 Measurement of lignin content

Plant material was lyophilized for 48h and ground to a fine powder. A first clean-up was done with 70% EtOH and followed by three subsequent clean-up steps with 1:1 MeOH: $CHCl_3$ and finally a finishing acetone wash step. Cleaned cell wall extracts (AIR) were dried 24h at room temperature to remove all excess solvents.

Lignin analysis was performed according to Fukushima and Hatfield (2005) using the acetylbromide soluble lignin (ABSL) method. 1-2mg of cleaned cell wall extracts was incubated with 100 µL of 25% (v/v) acetylbromide in glacial acetic acid at 50°C for 3 h with vortexing every 15 minutes. The reaction was stopped on ice and neutralized with 400 µL 2M NaOH, 70 µL freshly prepared 0.5 M hydroxylamine hydrochloride was added and vortexed. 1420 µL glacial acetic acid was added, samples mixed by inverting tubes and 200 µL pipetted into a UV specific 96 well plate. Samples read in an ELISA reader at 280nm and ABSL content calculated by following formula:

$$\%ABSL = \frac{(Abs_{280} \times 2\ ml \times 100\%)}{(Coeff \times pathlength \times weight\ (mg))}$$

Coefficient for grasses: 17.75 as described in literature. For the present study the path length of ELISA system was 0.539 cm.

7.2.3 Measurements of hordatines

Hordatine levels were measured in the second and third leaves of barley seedlings of wild-type and the two transgenic lines expressing *Lr34res*, with a method for sample preparation described by (De Vos et al., 2007). Second and third leaves of two different 18-day-old plants were pooled and five replicates were prepared per line. As internal standard, ampicillin was added to extraction solvent at a concentration of 0.25 mg l^{-1}. Supernatants were diluted 1:1 with water and 5 µl were injected for LC-MS analysis. Relative levels of hordatines were determined with an UHPLC system (Waters Acquity, Milford, USA) connected to an electrospray (ESI) quadrupole time-of-flight mass spectrometer (maXis, Bruker Daltonics, Bremen, Germany). Hordatines were separated at 40°C on a Waters Acquity BEH C18 column (1.7 mm, 2.1 x 100 mm), a flow rate of 0.4 ml min^{-1}, and with a mobile phases composed of water (A) and acetonitrile (B), both containing 0.1% [*v/v*] formic acid. The following gradient was used (proportion of solvent B): 3% during 0.5 min, 25% at 13 min, and finally flushed with 100% for 2 min. Mass spectra were recorded over a mass range m/z 50-1500 in

(+)-ESI. Calibration was achieved by sodium formate clusters, and extracted ion chromatograms were isolated with tolerance ± 0.01. Peak areas were normalized to ampicilline.

Hordatines were putatively annotated based on accurate mass (hordatine A: $[C_{28}H_{38}N_8O_4+2H]^{2+}$, 7.20 min, m/z_{meas} 267.15844, + 1.3 ppm, 7.87 min, m/z_{meas} 267.15841, + 1.2 ppm; hordatine B: $[C_{29}H_{40}N_8O_5+2H]^{2+}$, 6.90 min, m/z_{meas} 291.16354, + 0.6 ppm, 7.83 min, m/z_{meas} 291.16353, + 0.6 ppm). Furthermore, MS/MS spectra (CE 35-70 eV, isolation width 8) showed a high similarity to hordatines characterized with matrix-assisted laser desorption ionization (MALDI)-MS/MS in germinating barley seedling (Gorzolka et al., 2014). The two peaks found for hordatine A and B respectively showed identical MS/MS spectra and probably arose from cis/trans isomers (Wakimoto 2009).

7.3 Supplemental references

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Chapter C: Pathogen-inducible *Ta-Lr34res* expression in heterologous barley confers disease resistance without negative pleiotropic effects

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Summary

Plant diseases are a serious threat to crop production. The informed use of naturally occurring disease resistance in plant breeding can greatly contribute to sustainably reduce yield losses caused by plant pathogens. The *Ta-Lr34*res gene encodes an ABC transporter protein and confers partial, durable and broad-spectrum resistance against several fungal pathogens in wheat. Transgenic barley lines expressing *Ta-Lr34res* showed enhanced resistance against powdery mildew and leaf rust of barley. While *Ta-Lr34res* is only active at adult stage in wheat, *Ta-Lr34res* was found to be highly expressed already at the seedling stage in transgenic barley resulting in severe negative effects on growth. Here we expressed *Ta-Lr34res* under the control of the pathogen-inducible *Hv-Ger4c* promoter in barley. Sixteen independent barley transformants showed strong resistance against leaf rust and powdery mildew. Infection assays and growth parameter measurements were performed under standard glasshouse and near-field conditions using a convertible glasshouse. Two *Hv-Ger4c::Ta-Lr34res* transgenic events were analysed in detail. Plants of one transformation event had similar grain production compared to wild-type under glasshouse and near-field conditions. Our results showed that negative effects caused by constitutive high expression of *Ta-Lr34res* driven by the endogenous wheat promoter in barley can be eliminated by inducible expression without compromising disease resistance. These data demonstrate that *Ta-Lr34res* is agronomical useful in barley. We conclude that the generation of a large number of transformants in different barley cultivars followed by early field testing will allow identifying barley lines suitable for breeding.

1. Introduction

To ensure global food security is one of the top challenges in this century. The ever growing world population is the main driver for increasing demand for agricultural products until 2050 (Gerland et al., 2014). Loss of agricultural land and climate change are additional factors that require a higher productivity (Godfray et al., 2010). Plant pathogens are responsible for worldwide crop losses of 10-16% on average (Chakraborty and Newton, 2011; Oerke, 2006). A major strategy to improve the efficiency of crop production is to enhance plant resistance against diseases by taking advantage of the large diversity of naturally existing resistance genes. To do so, identified resistance genes can be transferred to other crop cultivars by classical breeding or introduced into other plant species by stable genetic transformation. Most resistance genes are specific to one pathosystem, but in a few cases resistance genes were shown to be functional in several plant-pathogen interactions. Maize *Rxo1* is an example of a resistance gene that was functionally transferred to the heterologous grass species rice where it mediates resistance against *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al., 2005). *Pto*, a resistance gene originating from tomato and conferring resistance to *Pseudomonas syringae* pv. tomato, was functional against strains of *P.s.* pv. *tabaci* expressing the effector *avrPto* after stable transformation into *Nicotiana benthamiana* (Rommens et al., 1995).

An interesting group of durable, multi-pathogen resistance genes against fungal pathogens has been specifically identified in hexaploid bread wheat (*Triticum aestivum*): It includes *Lr67* (=*Yr46/Sr55/Pm46*), *Lr46* (=*Yr29/Sr58/Pm39*) and *Lr34* (=*Yr18/Pm38/Sr57*). All these genes confer similar phenotypes in wheat, including a senescence-like phenotype in the flag leaf described as leaf tip necrosis (LTN) (Singh and Huerta-Espino, 1997) and partial disease resistance. *Lr67* and *Lr34* have been cloned and were found to encode a hexose transporter (Moore et al., 2015) and an ATP-binding cassette (ABC) transporter, respectively (Krattinger et al., 2009). The Lr34 and Lr67 proteins are not classical resistance proteins such as receptor-like kinases (RLKs), receptor-like proteins (RLPs) and nucleotide-binding, leucine-rich repeat receptor proteins (NLRs) (Krattinger and Keller, 2016)

and their molecular function is not yet fully understood. *Lr67*, *Lr34* and *Lr46* were shown to confer partial resistance against leaf rust (*Puccinia triticina*), stripe rust (*P. striiformis* f.sp. *tritici*), stem rust (*P. graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici, Bgt*) of wheat (Ellis et al., 2014; Spielmeyer et al., 2013).

Ta-Lr34 has been successfully used in wheat resistance breeding since the beginning of the last century (Kolmer et al., 2008). *Ta-Lr34* has two predominant alleles in the wheat gene pool, referred to as *Ta-Lr34res* (resistant) and *Ta-Lr34sus* (susceptible) (Dakouri et al., 2010; Lagudah, 2011). *Ta*-Lr34res and *Ta*-Lr34sus differ only by two amino acid polymorphisms and *Ta-Lr34res* evolved from the ancestral *Ta-Lr34sus* version after domestication (Krattinger et al., 2013). Orthologues of *Ta-Lr34res* were detected in rice and *Sorghum*, but not in maize and barley. Based on the two critical amino acid polymorphisms of *Ta*-Lr34res in wheat, all of these orthologues were identified as susceptible haplotypes (Krattinger et al., 2013; Krattinger et al., 2011).

Ta-Lr34res was transformed to rice (Krattinger et al., 2016), barley (Risk et al., 2013) and maize (Sucher et al., 2016). In all three grass species *Ta*-Lr34res was found to be functional: In rice it mediated resistance against rice blast (*Magnaporthe oryzae*), accompanied by a late LTN phenotype (Krattinger et al., 2016). In barley, *Ta-Lr34res*, but not *Ta-Lr34sus* provided strong resistance against barley leaf rust (*Puccinia hordei*) and barley powdery mildew (*Blumeria graminis f. sp hordei, Bgh*). In contrast to wheat, *Ta-Lr34res* under control of its native promoter was highly expressed already at seedling stage in barley, resulting in an early strong LTN phenotype. Thus, *Ta-Lr34res* activity is accompanied by drastic pleiotropic effects with high fitness costs in barley.

Reducing or eliminating negative effects of a transgene can be achieved by using a different genetic background or by altering the expression level. As the negative effects of *Ta-Lr34res* were described to be expression level-dependent (Chauhan et al., 2015), we tested whether altered *Ta-Lr34res* expression can result in barley plants with good levels of disease resistance but no negative effects on growth vigour. We hypothesize that to minimize negative effects on barley growth, *Ta-Lr34res* should only be active when resistance is needed, i.e. at the early stage of infection. In this case the

promoter of choice would be pathogen-inducible. Pathogen-inducible promoters are activated early after infection (Hernandez-Garcia and Finer, 2014). A well described pathogen-induced barley (*Hordeum vulgare*) gene is *Hv-Ger4c*, which belongs to a cluster of nine genes on barley chromosome 4H (Druka et al., 2002). Himmelbach et al. (2010) fused the *Hv-Ger4c* promoter to *GUS* and performed transient expression assays as well as experiments using stably transformed barley plants. Plants inoculated with *Bgh* and *Puccinia hordei* as well as the non-host pathogens *Bgt* and *Puccinia triticina* showed increased expression levels compared to non-infected controls, regardless if the interaction was compatible or not. Expression was measured in epidermal peels as well as whole leaf tissue. The results revealed an epidermis-specific expression pattern of the *Hv-Ger4c* promoter. Further, Himmelbach at al. (2010) reported that *Hv-Ger4c* is not activated upon abiotic stress treatment such as ozone, cold, wounding, UV irradiation or wind. This made the *Hv-Ger4c* promoter an excellent candidate for our study. Here, we provide proof of concept that induced, epidermis-specific expression of *Ta-Lr34res* in barley results in broad spectrum resistance without compromising fitness and this can provide much needed inbuilt resistance against fungal pathogens.

2. Results

2.1 Characterization of the barley lines transgenic for *Hv-Ger4c*::*Ta-Lr34res*

We stably transformed barley cv. Golden Promise with *Ta-Lr34res* under the control of the *Hv-Ger4c* promoter and characterized the resulting transgenic lines (later called *Hv-Ger4c*::*Ta-Lr34res* lines) for transgene expression, disease resistance and growth parameters. Nineteen primary transformants (T0) were obtained and grown to maturity. Eighteen T0 plants produced grains and T1 plants were genotyped by PCR. Plants were analysed regarding the development of LTN, resistance to the barley powdery mildew isolate K1 and the expression of the full-length *Ta-Lr34res* coding sequence (cDNA) (Supplemental table 1).

In order to identify potential transformants with only slightly reduced or unaffected fitness but good disease resistance, two-week-old T1 plants were analysed in a first step for the presence/absence of LTN. In addition, resistance to powdery mildew was analysed on three-week-old plants 7 days after infection (dpi). Progeny of sixteen events showed either strong LTN at seedling stage (six T1 progeny, Supplemental table 1) similar to line BG 9 with the native *Ta-Lr34res* promoter (Chauhan et al., 2015; Risk et al., 2013) or were susceptible (eight T1 progeny) and were therefore not of interest for further investigation. T1 progeny of events 8 and 11 showed no or reduced LTN compared to line BG 9. Both lines showed resistance to powdery mildew 7 dpi at a comparable level as line BG 9. For further analyses, progeny of events 8 and 11 were selected for uniform resistance to powdery mildew in T2. To do so, at least 24 T2 seedlings of different individual T1 plants were grown to two-week-old plantlets and infected with barley powdery mildew. T2 families showing uniform resistance and no segregation pattern in the genotype based on PCR were considered as uniform lines. Southern blot analysis (based on a probe derived from the marker gene used in transformation) of these T2 lines, uniform for resistance and LTN phenotype, detected three non-segregating insertions and one segregating insertion in line 8 and two insertions of which one segregated in line 11 (Supplemental figure 1). In both lines 8 and 11, the segregating insertion had no influence on the

resistance phenotype, indicating that uniform lines are uniform for the functional insertion(s) represented by the non-segregating bands in the Southern blot (Supplemental figure 1). Therefore, seeds of uniform lines were pooled within transformation events and used for further investigation. Similarly, the corresponding azygous sister lines (null-segregants) were determined.

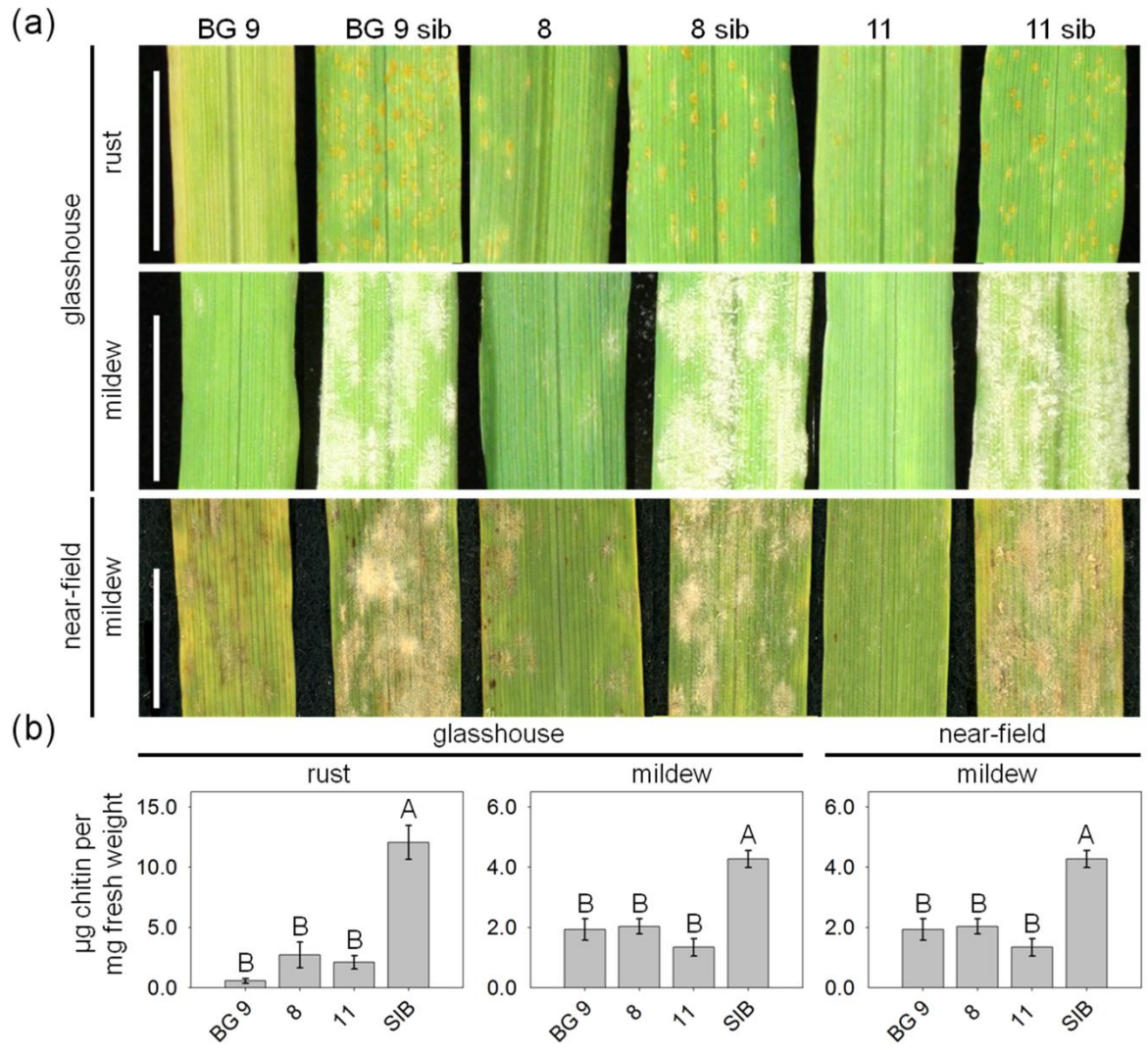

Figure 1: *Hv-Ger4c::Ta-Lr34res* transgenic barley shows *Ta-Lr34res*-mediated disease resistance.

For assessment of *Ta-Lr34res*-mediated resistance under standard glasshouse conditions the fourth leaves of plants at the five-leaf stage were taken 7 dpi. For the assessment of resistance under near-field conditions, fourth leaves of six-leaf stage plants were evaluated. Leaves were used for macroscopic observations (a) as well as chitin measurement (b). Columns show the amount of chitin in µg chitin per mg fresh weight as an average of 3-7 biological replicates. Samples of all azygous sister lines were pooled (SIB). Error bars indicate standard errors. Scale bars represent 1 cm. Statistical analysis was performed on square root-transformed (rust, glasshouse) or non transformed (near-field) values using the all pairs Tukey-Kramer HSD test. In case of mildew, glasshouse, Kruskal-Wallis test on non-transformed values was performed. Levels not connected by the same letter are significantly different (p-values: < 0.02).

2.2 *Hv-Ger4c*::*Ta-Lr34res* lines showed enhanced resistance to barley leaf rust and powdery mildew

To further characterize pathogen resistance, fourth leaves of T2 plants of the lines 8, 11, BG 9 and their corresponding azygous sister lines were taken for macroscopic and biochemical analyses of *Ta-Lr34res*-mediated resistance. We performed infection assays with the barley leaf rust isolate BRG1.2.1 and the barley powdery mildew isolate K1 as described in Risk et al. (2013). Line BG 9 developed no visual symptoms upon infection with barley leaf rust while the corresponding azygous sister line BG 9 sib was fully covered by uredia (Figure 1a). In line 8, a much lower amount of uredia developed compared to the azygous sister line 8 sib. In addition, uredia were smaller and surrounded by chlorotic flecks (Figure 1a), mimicking the partial resistance in wheat. In line 11, mainly chlorotic spots and a few very small uredia were observed. Whereas the corresponding azygous sister line 11 sib was fully covered by uredia, indicating full susceptibility (Figure 1a). In the infection assay using barley powdery mildew, line BG 9 showed only a few chlorotic spots and both lines 8 and 11 developed only a few mildew pustules, while all azygous sister lines were completely covered by the fungus (Figure 1a). To assess if resistance of the barley lines transgenic for *Ta-Lr34res* under the control of the *Hv-Ger4c* promoter might be of interest for agricultural application, an infection assay was performed under near-field conditions. To do so, the same lines tested in the glasshouse were grown in a convertible glasshouse (Romeis et al. 2007) and artificially infected with barley powdery mildew. Under these near-field conditions similar macroscopical results were observed regarding resistance to powdery mildew as recorded in the glasshouse, indicating the functionality of the *Hv-Ger4c*::*Ta-Lr34res*-mediated resistance under near-field conditions (Figure 1a). To quantify *Ta-Lr34res*-mediated resistance, the amount of the fungal cell wall component chitin was measured using the biochemical WGA-FITC method (Ayliffe et al., 2014; Ayliffe et al., 2013). The fourth leaves of plants at the five-leaf stage were taken in the glasshouse experiment whereas for the experiment under near-field conditions fourth leaves at the six-leaf stage were used for chitin measurements.

In the infection assay in the glasshouse using barley leaf rust, a Kruskal-Wallis test analysis indicates that overall differences in the level of chitin exist among lines (X^2(3, N=42)=31.2, p<0.001). Post-hoc comparison tests showed that there was a significantly reduced chitin amount in all *Hv-Ger4c*::*Ta-Lr34res* lines compared to the pooled azygous sister lines (all pair-wise p-values between transgenic lines and the azygous sister lines <0.008, Figure 1b). Further, chitin levels of lines 8 and 11 were not different from the one in line BG 9 (pair-wise p-values >0.5, Figure 1b). After infection with barley powdery mildew in the glasshouse, a Kruskal-Wallis test analysis revealed overall differences among lines (X^2(3,N=42)=29.8, p<0.0001). Here again, post-hoc pair-wise comparison tests revealed that chitin amounts were clearly reduced compared to the pooled azygous sister lines (all pair-wise p-values <0.02) and chitin amounts of lines 8 and 11 were similar to the one in BG 9 (all pair-wise p-values >0.5).

Under near-field conditions (Kruskal-Wallis test, (X^2,(3,N=42)=26.5,p<0.0001) all *Hv-Ger4c*::*Ta-Lr34res* lines showed a significant reduction in chitin amounts compared to the azygous sister lines (all pair-wise p-values <0.02) and reached similar chitin levels as the highly expressing line BG 9 (all pair-wise p-values >0.9, Figure 1b). Taken together, these results show that all *Hv-Ger4c*::*Ta-Lr34res* lines reach similar resistance levels as the highly expressing line BG 9 where the *Ta-Lr34res* gene is under control of its own promoter.

2.3 *Hv-Ger4c*::*Ta-Lr34res* lines show reduced LTN and reduced impact on growth parameters

To investigate if the inducible *Hv-Ger4c*::*Ta-Lr34res* lines show less negative effects on growth parameters compared to BG 9, seedlings were investigated for the presence and intensity of LTN. Whereas line 8 showed no LTN in the first leaf at the five-leaf stage, line 11 showed LTN, but at a reduced level compared to BG 9 under glasshouse conditions (Figure 2a). The reduced LTN level at the five-leaf stage might indicate an overall reduction in fitness costs. To determine possible negative effects of *Hv-Ger4c*::*Ta-Lr34res* at later growth stages, plants were grown until maturity and growth parameters were measured. Growth parameters included gram of grains per plant, dry

weight, number of spikes per plant, number of grains per spike, grains per plant and the 1,000-grain weight. In order to test the influence of environmental conditions on growth parameters, data were collected from plants grown in the glasshouse and under near-field conditions, respectively (Figure 2 and Supplemental figure 2).

Under glasshouse conditions, lines 8 and 11 reached similar values in gram of grains per plant as their corresponding azygous sister lines (ANOVA ($F(5,54)=41.6$, p-value $<2e^{-16}$), all pair-wise p-values >0.2, Figure 2b). Furthermore, for both lines similar values were measured compared to their azygous sister lines for all growth parameters tested (Supplemental figure 2 and Supplemental table 2). Lines 8 and 11 showed considerably higher values than BG 9 in all measurements, indicating an improvement of growth vigour by the use of the *Hv-Ger4c* promoter to control *Ta-Lr34res* expression (Figure 2b and Supplemental figure 2).

We also wanted to test if altered expression of *Ta-Lr34res* by the *Hv-Ger4c* promoter would lead to improved growth vigour under near-field conditions. All *Hv-Ger4c*::*Ta-Lr34res* lines showed no LTN phenotype compared to BG 9 at the seedling stage (Figure 2a). Line 8 performed the same as the azygous sister line for all growth parameters, indicating that *Ta-Lr34res* was not linked to negative effects in this particular line (Figure 2b, Supplemental figure 2 and Supplemental table 2). At later stages, the negative effects on growth developed continuously in lines BG 9, 11 and 11 sib, resulting in lower values for gram grains (Figure 2), number of spikes per plant and grains per plant (Supplemental figure 2). Lines BG 9, 11 and 11 sib produced less dry weight biomass than line 8, line 8 sib and line BG 9 sib. This resulted in a lower number of spikes per plant and consequently a lower amount of grains per plant (Supplemental figure 2 and Supplemental table 2). In addition, lines BG 9, 11 and 11 sib lodged at the flowering stage. They produced grains on ground-lying tillers, which lead to 1,000-grain weights which were mostly not different compared to their corresponding azygous sister lines (Supplemental figure 2 and Supplemental table 2). Because of the broken tillers, lines BG 9, 11 and 11 sib turned out not to have appropriate growth vigour under near-field conditions. In most cases, growth values of line 11 did not differ from line 11 sib. This indicates that the negative

effects observed for this line under near-field conditions must have been caused by somaclonal or epigenetic variation due to the tissue culture process rather than by the presence of *Ta-Lr34res*.

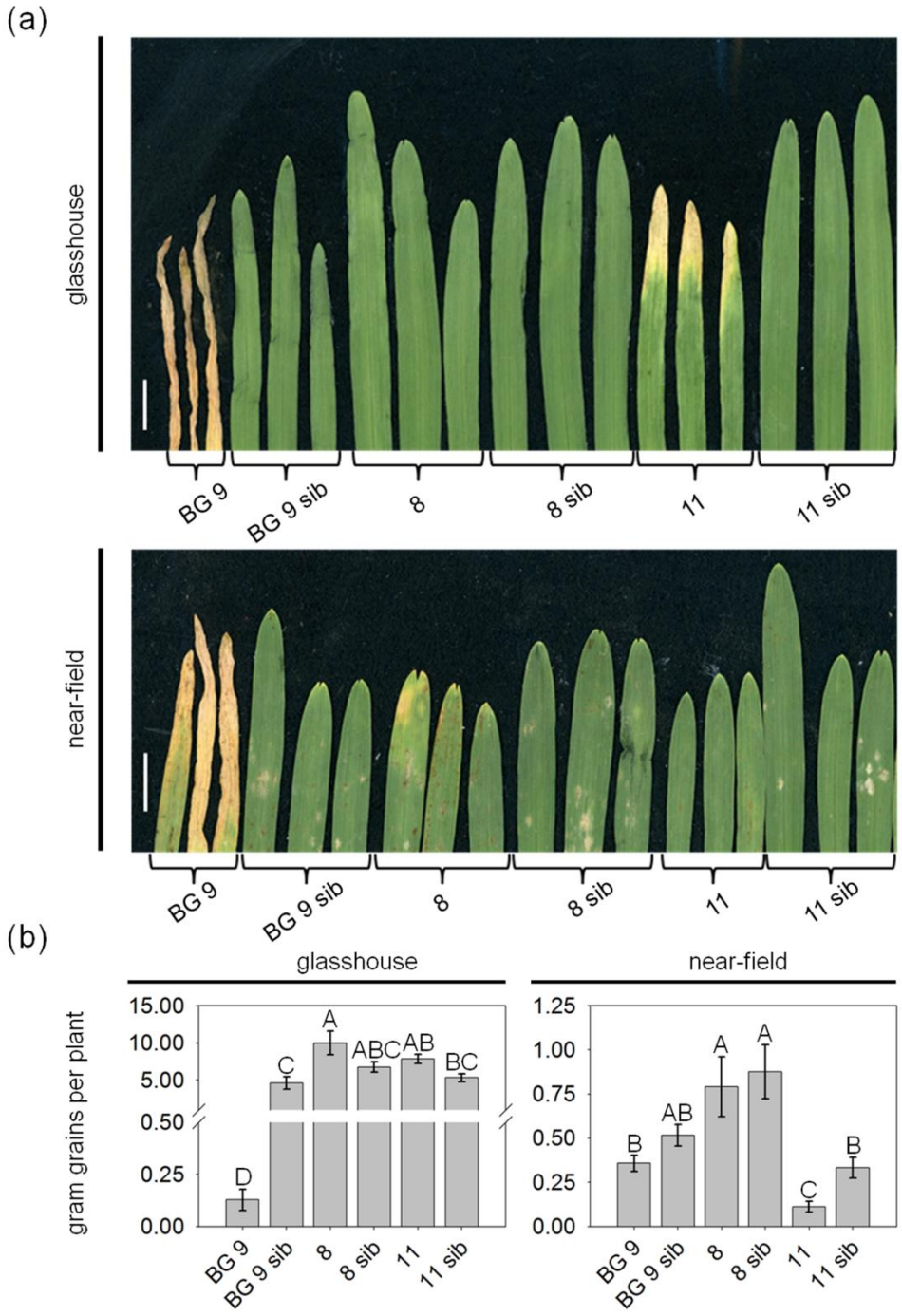

Figure 2: *Hv-Ger4c::Ta-Lr34res* barley lines show reduced LTN and reduced impact on growth parameters.
(a) For the assessment of LTN, the first leaf of plants at the five-leaf stage was taken. (b) For assessment of growth parameters, plants were grown until maturity, 10 individual plants were harvested and gram grains per plant were determined. Plants under standard glasshouse conditions were 138 days, plants under near-field conditions were 140 days old. Scale bars represent 1 cm. Error bars indicate standard errors. Statistical analysis was done on square root-transformed values using the all pairs Tukey-Kramer HSD test. Levels not connected by the same letter are significantly different (p-values: < 0.05).

2.4 Induction of *Ta-Lr34res* expression by the *Hv-Ger4c* promoter

ANOVA analysis of expression data revealed significant differences of *Ta-Lr34res* expression among the tested lines (rust glasshouse: ($F(5,32)=47.9$, p-value <0.0001, Figure 3a), mildew glasshouse: ($F(4,28)=93.2$, p-value<0.0001, Figure 3b) near-field: ($F(2,18)=42.6$ p-value<0.0001, Supplemental figure 4). Expression analysis revealed that the *Ta-Lr34res* expression levels of the uninfected *Hv-Ger4c*::*Ta-Lr34res* lines were 12 to 39-fold lower compared to the uninfected line BG 9 carrying the *Ta-Lr34res* promoter (Figure 3) under glasshouse conditions. A low level of basal expression in uninfected plants of line 8 was detectable by RT-qPCR in the rust assay but not in the mildew experiment. This is possibly due to the different plant growth conditions used in the mildew and rust experiments. Expression levels under near-field conditions of uninfected *Hv-Ger4c*::*Ta-Lr34res* plants were 5 to 11-fold lower compared to BG 9 (Supplemental figure 4).

To assess if *Ta-Lr34res* expression was pathogen-inducible after fusion to the *Hv-Ger4c* promoter, infection assays with powdery mildew and barley leaf rust were performed. Fourteen-day-old seedlings were inoculated with barley powdery mildew and barley leaf rust, respectively. After 7 dpi (mildew) and 10 dpi (rust), respectively, third leaves were harvested for expression analysis. Infected leaves were compared to mock-infected leaves grown at the same time and under the same conditions. Interestingly, no induction upon rust infection was detected (all pair-wise comparison between mock- and infected lines p-values >0.7, Figure 3a). In contrast, *Ta-Lr34res* expression was induced upon infection with powdery mildew in all *Hv-Ger4c*::*Ta-Lr34res* lines (line 11: pair-wise p-value <0.0001). Expression in line 11 was 4-fold higher upon infection. In line 8, the expression level upon mock infection was below detection level and therefore no statistical analysis was possible. Yet, expression of *Ta-Lr34res* was detectable in infected line 8 and reached similar expression levels as infected line 11 (pair-wise comparison p-value =0.86), indicating that *Ta*-Lr34res was also induced upon infection in line 8 (Figure 3b).

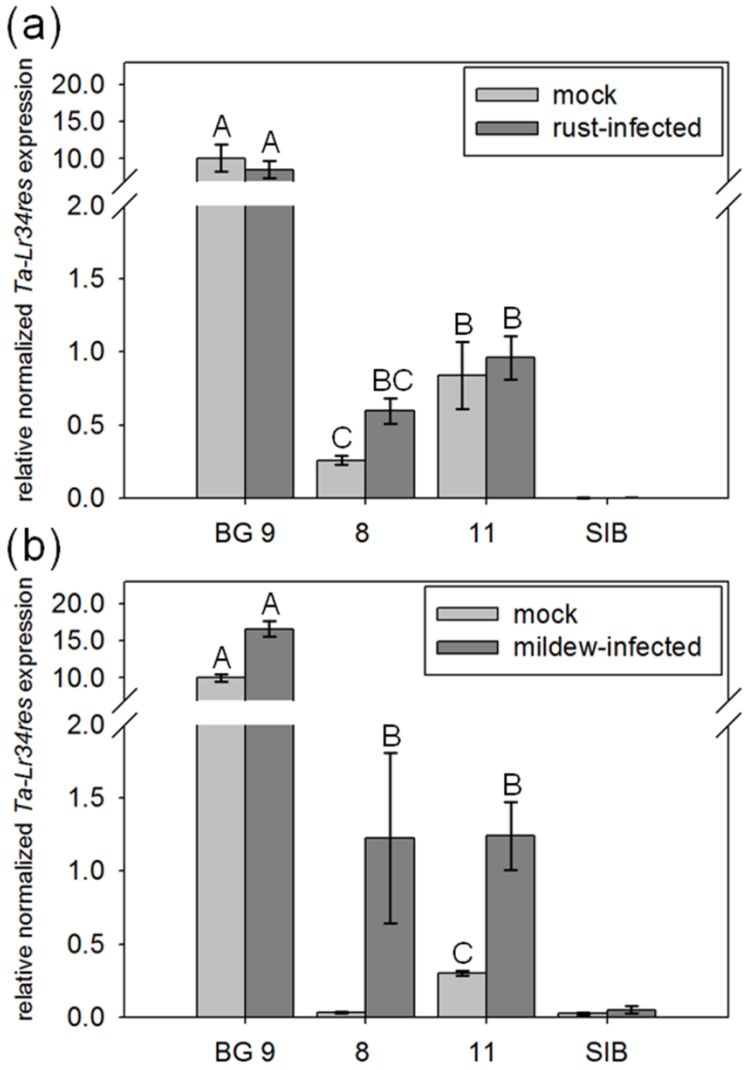

Figure 3: Expression analysis of *Ta-Lr34res*.

The second leaf of nineteen-day-old plants, 9 dpi rust (a) and powdery mildew (b), were harvested for RNA extraction and expression analysis by RT-qPCR. Bars show the relative *Ta-Lr34res* expression normalized to *GAPDH* as an average of 4-7 biological replicates. Basal expression level of line 8 was below detection level in the mildew experiment possibly due to different growth conditions between the rust and mildew infection experiment. SIB represents the average of all azygous sister lines representing the negative control. Error bars represent standard errors. Statistical analysis was done on log10-transformed expression values using the all pairs Tukey-Kramer HSD test. Levels not connected by the same letter are significantly different (p-value: < 0.001).

3. Discussion

Enhancing resistance by alteration of expression levels is frequently accompanied by increased fitness costs. The over-expression of tomato *Pto* (Tang et al., 1999) or of the *NLR* gene *BAL* in *Arabidopsis* (Stokes et al., 2002) led to microscopic lesions or dwarfing and twisted leaves, respectively. Furthermore, wheat lines over-expressing the *NLR* gene *Lr10*, showed reduced grain weight compared to the *Lr10* donor line under near-field conditions (Feuillet et al., 2003; Romeis et al., 2007). Interestingly, different pleiotropic effects caused by the same transgene have been observed among different transformation events. In one of four transgenic lines over-expressing the wheat powdery mildew disease resistance gene *Pm3b*, no pleiotropic effects were observed under field conditions. In the three lines Pm3b#2-4, plants showed different level of chlorosis and reduced fertility, whereas this phenomenon was not observed in line Pm3b#1 (Brunner et al., 2011). The different phenotypes of the two genotypes Pm3b#2 and Pm3b#1 could be explained by higher *Pm3b* expression levels in Pm3b#2 compared to Pm3b#1. However, Pm3b#1 and Pm3b#4 showed similar expression levels of *Pm3b* transcripts but no pleiotropic effects were detected in Pm3b#1, indicating that not only expression levels but also position effects with subtle changes in expression over time and space might influence the phenotype (Brunner et al., 2011).

In the present study, we found that one of two lines transgenic for *Hv-Ger4c*::*Ta-Lr34res* analysed in detail (line 8), showed enhanced disease resistance without negative pleiotropic effects under glasshouse and near-field conditions. This demonstrates that the *Ta-Lr34res* gene can be used as a resistance source in barley, very similarly to wheat.

The only pathogen-inducible barley promoter with demonstrated functionality in driving conditional expression of a transgene is *Hv-Ger4c* (Himmelbach et al., 2010). Although we found this promoter to be highly useful in our work, it might be worth exploring additional inducible promoters for fine-tuning expression levels. It has been shown that pathogen- and defence hormone-inducible promoters can be functional in heterologous species, as shown for *Os-PR10a* (induced by

Xanthomonas oryzae pv *oryzae* and salicylic acid, SA), which was activated by SA in stably transformed *Arabidopsis* (Hwang et al., 2008). Together with the rapid accumulation of RNAseq data that allow a fast identification of pathogen-inducible genes, there is an increasing number of candidate promoters that can be used for analysis of inducible expression.

In this study, two lines expressing *Ta-Lr34res* under control of the *Hv-Ger4c* promoter were selected for detailed analyses. There, we detected some basal expression of *Ta-Lr34res* in the *Hv-Ger4c*::*Ta-Lr34res* lines and *Ta-Lr34res* was specifically induced by the *Hv-Ger4c* promoter after infection with powdery mildew, but not with barley leaf rust. Similarly, Himmelbach et al. (2010) measured a lower induction of *Hv-Ger4c* promoter-driven *GUS* expression after infection with barley leaf rust compared to powdery mildew. This could be due to the fact that barley powdery mildew infects epidermal cells, whereas barley leaf rust grows mainly in the mesophyll. Possibly, this indicates differences in inducibility of the promoter by pathogens in different tissues of barley. The fact that *Hv-Ger4c*::*Ta-Lr34res* lines showed resistance against barley leaf rust although an induction of *Ta-Lr34res* expression was not confirmed, indicates that the detected basal expression of *Ta-Lr34res* was sufficient to mediate disease resistance.

The combination of different resistance genes is generally considered to be a successful strategy to enhance resistance. In barley, a considerable number of resistance genes have been described (Andersen et al., 2016; Seeholzer et al., 2010). The most well-known and cloned major barley resistance genes that act efficiently as single genes are *Rpg1* and the *Mla* alleles (Brueggeman et al., 2002; Chelkowski et al., 2003). It has been shown that the use of quantitatively acting resistance genes can extend the life-expectancy of *R* genes and the combination of these two types of resistance is a very promising approach to achieve durable resistance in agricultural ecosystems (Brun et al., 2010; McDonald, 2010). Thus, a combination of e.g. *Mla* genes with the quantitatively acting *Ta-Lr34res* might be an effective strategy to achieve durable resistance against mildew in barley. Another quantitatively acting gene in barley mildew resistance is the modulator of defence

and cell death *Mlo* gene, which encodes a seven-transmembrane domain protein and is a negative regulator of pathogen defence response. Homozygous loss-of-function mutant (*mlo*) barley plants show enhanced and durable resistance against barley powdery mildew (Jorgensen, 1992). So far, several mutant alleles (*mlo*) were described which resulted in inhibited fungal growth (Buschges et al., 1997; Piffanelli et al., 2002).

The *Rpg1* gene encodes a protein with two tandem kinase domains and has been used in the northern parts of the USA and Canada to protect barley from stem rust for more than 65 years (Brueggeman et al., 2002). Therefore, *Rpg1* confers durable resistance but in contrast to *Ta-Lr34res* this is a resistance active specifically against stem rust. It would be interesting to cross the *Hv-Ger4c*::*Ta-Lr34res* lines with Morex, the donor of *Rpg1*, to test if the *Rpg1*x*Ta-Lr34res* gene combination would result in still improved resistance compared to the corresponding parental lines. Pyramiding different resistance genes or alleles seems promising in order to enlarge spectrum and durability of pathogen resistance. However, stacking of certain different *Pm3* alleles resulted in suppression of the *Pm3*-mediated resistance response, indicating that combining of resistance genes and/or alleles is not always useful (Stirnweis et al., 2014).

Earlier studies demonstrated that *Ta-Lr34res* is transferrable to different crop species and was shown to confer durable, partial resistance also in these heterologous species (Chauhan et al., 2015; Krattinger et al., 2016; Rinaldo et al., 2016; Risk et al., 2013; Sucher et al., 2016). Interestingly, the expression of *Ta-Lr34res* was accompanied by negative pleiotropic effects, leading to increased fitness costs in barley and certain rice lines but not in durum wheat and maize. The data of this study and the results presented for barley by Chauhan et al. (2015) show a correlation of *Ta-Lr34res* expression and the level of disease resistance as well as negative pleiotropic effects in barley. Similar results have been reported in rice (Krattinger et al., 2016). In all rice lines transgenic for *Ta-Lr34res*, expression of the gene was controlled by its native promoter and Krattinger et al. (2016) identified one low-expressing transgenic rice line with no negative pleiotropic effects. This indicates that the

genetic backgrounds of rice, maize and durum wheat are all suitable to express *Ta-Lr34res* under the control of its own promoter to give good resistance without fitness costs, at least in some carefully selected genotypes. In contrast, in barley no *Ta-Lr34res* transgenic line under control of the native wheat promoter showed expression levels of *Ta-Lr34res* which were low enough to avoid negative pleiotropic effects. The genetic background of barley seems not to be suitable to allow optimal *Ta-Lr34res* expression levels when controlled by the native wheat promoter. Our results show that the negative pleiotropic effects of *Ta-Lr34res* in barley can be overcome by induced expression of *Ta-Lr34res* using the *Hv-Ger4c* promoter. Therefore, barley plants transgenic for *Ta-Lr34res* are potentially useful for agricultural applications. It seems rather straightforward to generate a large number of *Hv-Ger4c*::*Ta-Lr34res* lines or cross line 8 to elite barley cultivars and to select from them the lines with good resistance levels but no pleiotropic effects as determined under field conditions. Such lines would then represent ideal parents in breeding programmes.

4. Experimental procedures

4.1 Cloning of *Hv-Ger4c* promoter upstream of genomic fragments of *Ta-Lr34res* and stable transformation in barley

The Gateway binary vector p6UGLPΔSwaI containing the *Hv-Ger4c* promoter was used as a source for the promoter sequence (Himmelbach et al., 2010). The native promoter of *Ta-Lr34res* in constructs p6U:*Lr34*-ResGenomic (Risk et al., 2013) was replaced by a two way cloning strategy. First the full-length cDNA of *Ta-Lr34res* was cloned in the p6UGLPΔSwaI by Gateway cloning method resulting in the p6UGLPΔSwaI:*Lr34res*cDNA plasmid. There is a unique *Nru*I restriction site within the very first exon of *Ta-Lr34res* and a *MluI* site is present just upstream of *Ta-Lr34res* native promoter in p6U:*Lr34*-ResGenomic. The fragment containing *Hv-Ger4c* promoter and part of *Ta-Lr34res* cDNA till the *NruI* site was amplified with primers *MluI-proGLP*-Fwd (5'-ACGCGTCTGCAGGAATTCGATCAGC-3') and *NruI-Lr34*-Rev (5'- CGCTGTCGCGATGCCAATTCTAACTCGG-3') and cloned in PCR cloning vector pSCB (Agilent Technologies, USA) resulting in the pSCB:MluIProGLP-*Lr34*NruI plasmid. After confirmation by sequencing, the promoter *Hv-Ger4c* flanked by the *MluI* site and *Ta-Lr34res NruI* fragment was replaced in the original p6U:*Lr34*-ResGenomic with *MluI* and *NruI* restriction sites thereby exchanging the native *Ta-Lr34res* promoter with pathogen inducible *Hv-Ger4c* promoter.
Stable transformation of barley cv. Golden Promise was performed using *Agrobacterium*-mediated stable transformation according (Hensel et al., 2009) with the exception that the callus-forming media contained 1.25 mg/l $CuSO_4$ pentahydrate and the *A. tumefaciens* strain AGL-1 was used.

4.2 Selection of transformants uniform for resistance to powdery mildew

T0 plants were grown to adult stage and T1 grains were harvested. Two-week-old T1 seedlings were infected with powdery mildew isolate K1 and analysed macroscopically for *Ta-Lr34res*-mediated resistance (7 dpi). To do so, the barley line BG 9, transgenic for *Ta-Lr34res* under the native promoter and its corresponding sister line BG 9 sib (described earlier, (Risk et al., 2013) were used as

positive and negative control, respectively. Resistant T1 progeny were then checked for presence of *Hv-Ger4c*::*Ta-Lr34res* on genomic DNA level by PCR and analysed for full-length cDNA expression (Supplemental table 1). Finally, copy number was determined by southern blot analysis.

4.3 RNA extraction

Total RNA was extracted from 3rd leaves using SV Total RNA Isolation system (Promega, USA) and RNA integrity was checked by electrophoresis on a 1.2% agarose gel in 1x sodium-borate buffer. For cDNA synthesis RNA concentration was measured with a Nanodrop ND-1000 spectrophotometer and 500 ng of total RNA was used for cDNA synthesis with the iScriptTM advanced cDNA synthesis kit (Bio Rad, USA).

4.4 Detection of transgenic barley and determination of transgene copy number and full length cDNA amplification

Total genomic DNA was extracted from leaves using the CTAB method (Stein et al., 2001) and presence of *Ta-Lr34res* was assessed using the marker *csffr1* (Lagudah et al., 2009). Southern blot was performed by digesting 10 µg of genomic DNA with *EcoRI* and using a ^{32}P-labelled probe covering the *HPT* gene of the p6U vector as described previously (Risk et al., 2013). Segregants of the T1 generation negative in PCR were propagated to T2 and checked by Southern blot analysis. If absence of *Ta-Lr34res* was confirmed, they were considered to be azygous sister lines (sib).

To check for full-length cDNA expression, RNA was transcribed to cDNA using the M-MLV reverse transcriptase according to the manufacturers protocol (Invitrogen, USA) and a poly-T (30) oligo. The 4,319bp amplicon was amplified using the Phusion® High-Fidelity DNA Polymerase (New Englands BioLabs; USA) and a Touch-down PCR protocol (1. 98°C 3min, 2. 98°C 10sec, 3. 67°C 20sec, 72° 2min 50sec, 4. go 10x to 2. while reducing annealing temperature 1°C per cycle, 5. 95°C 10sec; 6. 55°C 20sec, 7. 72°C 2min 50sec, go 25x to 5, 8. 72° 5min). The primers used were FLC_F (5'GAGTACGGCTAGGCAATAGC3') and FLC_RW (5'GGCAAGTAGCTATATCTGTAAC3') whereby FLC stands for "full-length cDNA".

4.5 Determination of *Ta-Lr34res* gene expression by RT-qPCR

Expression was determined by RT-qPCR using a CFX96 or a CFX384 Touch Real-time PCR machine (Bio Rad, USA). Reactions were set up with 5 µl KAPA SYBR fast qPCR mix (KAPA biosystems, USA), *Ta-Lr34res* primer (500nM final concentration) (Risk et al., 2012) and 4 µl of 1:20 diluted cDNA per sample. *GAPDH* (Primers: *GAPDH*_Fw 5'CCGGGTTCCCACTGTGGAT3' and *GAPDH*_Rw 5'TGACTAGCAACTCGGTGCGG3'; E=102.0% R^2=0.977 Slope=3.466 y-int=21.012) and *ADP* (Gimenez et al., 2011) were used as reference genes. For each run, four technical replicates were used and four to seven biological replicates were used in all experiments.

4.6 Growth conditions and infection assays

Grains were sown in 96-well Jiffy pots filled with soil (Einheitserde Profi Substrat, Einheitserde Werkverband e.v., Germany) and put over night at 4°C in the dark for synchronized germination. Afterwards, plants were transferred to a growth chamber (16 h, 20°C, light and 8 h, 17°C, darkness) until indicated time points of the respective experiments. Seedling propagation was done by repoting plants to 2 l pots (one plant per pot) and growing them in glasshouse at standard growth conditions.

The convertible glasshouse system was described in (Romeis et al., 2007). Its roof is automatically opened during dry and windless weather conditions. In addition, one side is permanently open which allows outdoor temperatures (Supplemental figure 5). Twenty plants each of lines BG 9, BG 9 sib, lines 8, 8 sib, 11 and 11 sib were sown on 8th of March 2016 in central cylinders (42.2 litres) within big plastic containers filled with fresh farm soil which was taken freshly from a neighbouring field. Plants were grown until the five-leaf stage and reduced to ten plants per central cylinder. Five cylinders were used per line. The surrounding area was planted with Golden Promise (GP) as buffering plants. Plastic containers were arranged in two blocks and within each block each line was assigned randomly to a cylinder. The growth parameter measurements under glasshouse conditions

were performed in 15 l pots instead of cylinders in a similar manner with the exception that due to a lack of space no plastic containers with surrounding buffering plants were grown.

Barley leaf rust infections were performed as described in Risk et al. (2012) using spore suspension of the barley leaf rust isolate BRG1.2.1 in 3M_ Fluorinert_ FC-43 (3M, Switzerland). Rust infected plants were grown in a Conviron BDW80 chamber (Conviron, Canada) (16 h, 20°C, light and 8 h, 17°C, darkness). Mock infiltrations were also performed by spraying plants with FC-43 without spores. Powdery mildew infection was done by shaking the spores of isolate K1 from a pot containing approximately 25 infected plants (21 dpg) of 7 dpi barley cv. Golden Promise. Mock infections of the mildew experiments were performed by keeping a similar plant set under the same growth conditions without previous infection. Mildew infected plants were grown in a Sanyo MLR351 incubator (Sanyo, Japan) (16 h light, constantly 20°C). Artificial powdery mildew infections in the convertible glasshouse were done by potting two single plants (21 dpg, 14 dpi, infection performed in the lab) to the opposite edges of the surrounding area containing buffer plants at the time point when the plants in the cylinder reached the two leaf stage.

4.7 Chitin measurement

Chitin measurement was performed as described in Ayliffe et al. (2013 and 2014) with the following exceptions. Samples were diluted 1:10 for measurement; samples were treated for 20 min a steam cooker instead of autoclaving; the fluorometer Synergy H1 Hybrid 311 Reader (BioTek Instruments GmbH, Switzerland) was used for measuring. Four technical replicates were used for each sample. The readouts were analyzed using the software Gen5, version 2.03.1 (BioTek Instruments GmbH, Switzerland). Chitin amounts were calculated using standard curves (Supplemental figure 3) generated by defined amounts of Chitin from shrimp shells (Sigma-Aldrich, USA).

4.8 Statistical analysis

Differences in µg chitin per mg fresh weight and differences growth parameter values per plant across lines were tested using an ANOVA. If necessary, data were square root transformed to ensure normal distribution of residuals. When the normal distribution could not be reached, Kruskal-Wallis test were used. Multiple comparison p-values were then computed using the Post-hoc Tukey-Kramer HSD or Kruskal-Nemenyi tests. Expression values were $\log_{10}$-transformed to correct the exponential character of RT-qPCR. Differences in expression were tested with an ANOVA, following the same procedure as described above. All statistical analyses were performed in R v.3.2.1. Critical p-values were used for the decision for significance to create the letter codes. Critical p-values, p-values for all graphs shown and the transformation of raw data are given in Supplemental table 2.

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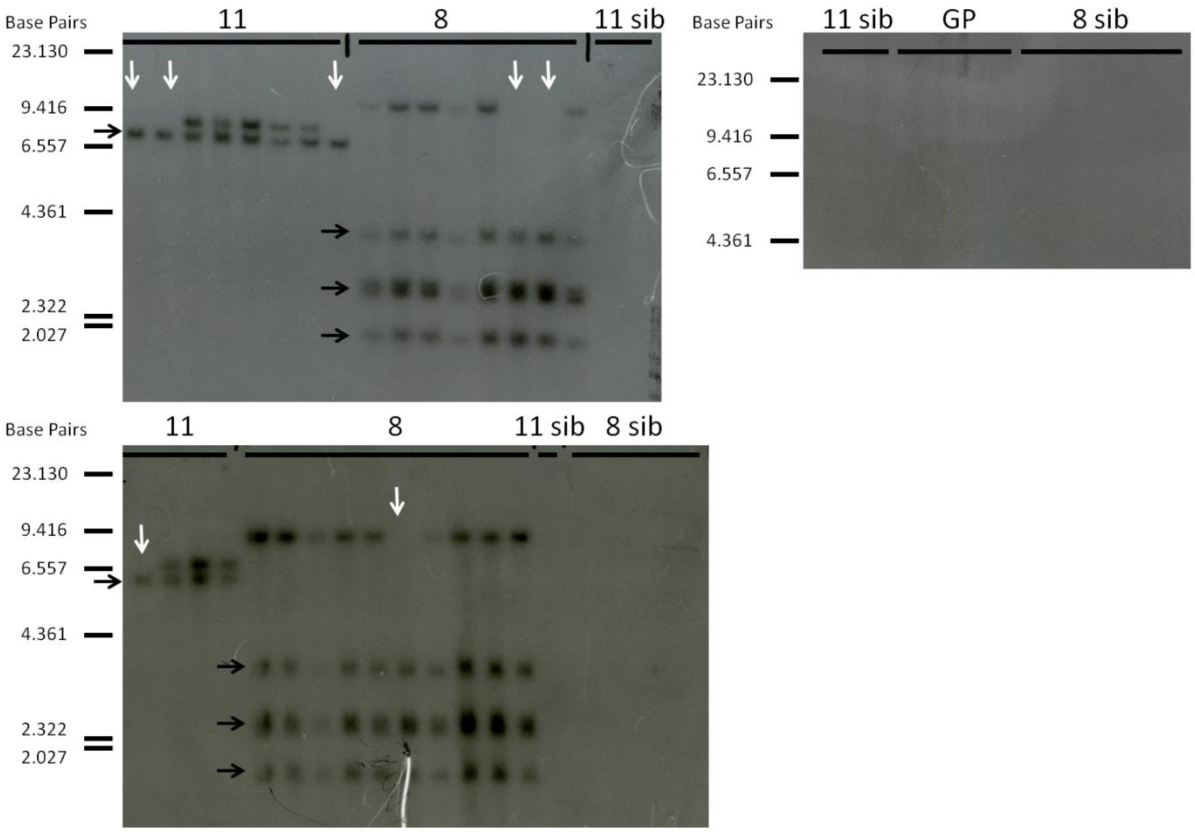
7. Supplemental material

Supplemental table 1: Phenotypical analysis of T1 progeny from different transformation events.

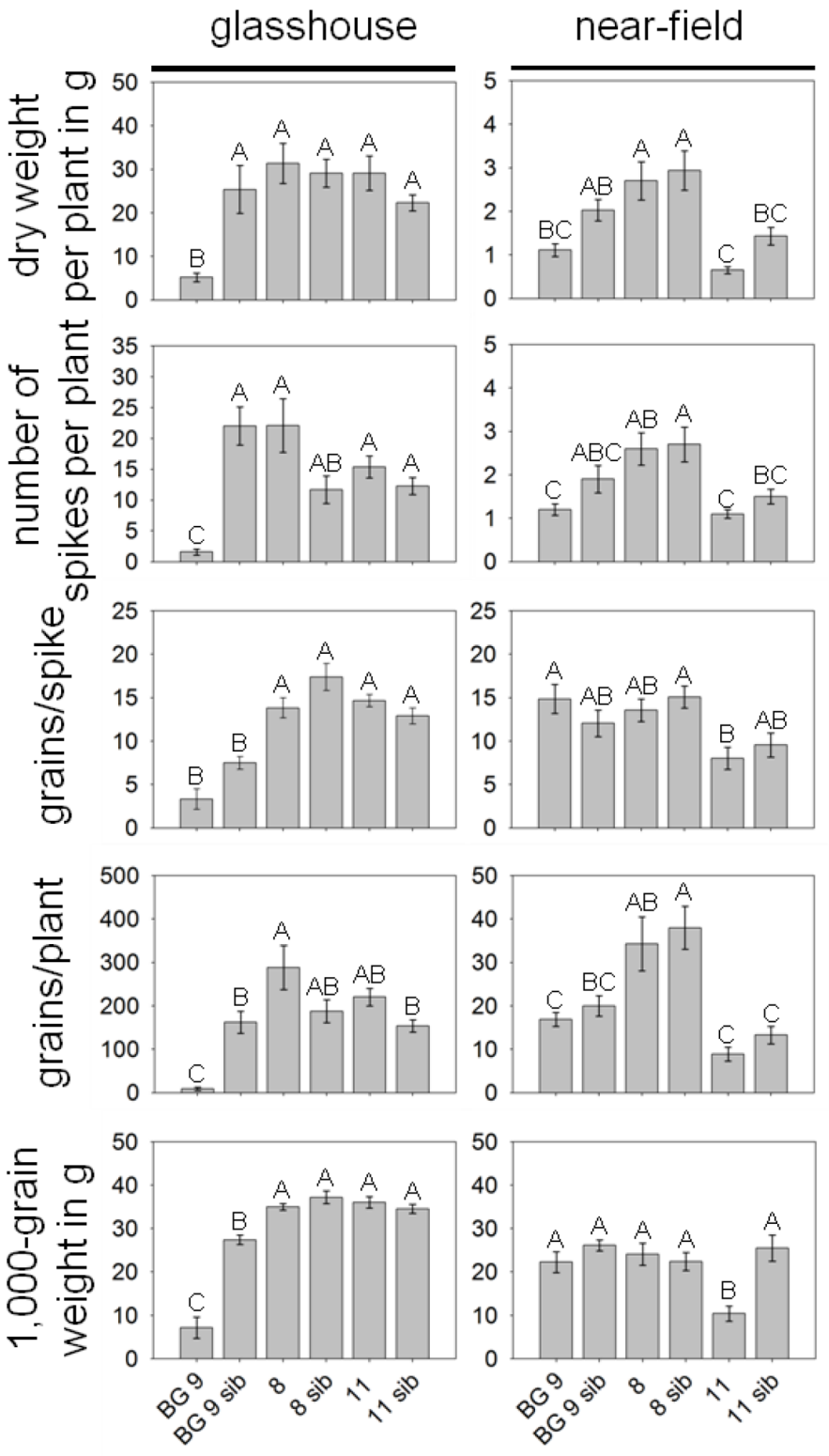
Two-week-old T1 plants were assessed for the presence/absence of LTN and infected with the barley powdery mildew isolate K1. 7 dpi, plants were macroscopically analyzed for resistance by presence/absence of infection symptoms. Later, full-length cDNA expression was studied by amplifying the complete 4,319bp cDNA fragment using PCR. The T0 plant of transformation event 1 produced no grains and is therefore not listed. Progeny of transformation events 8 and 11 (indicated in yellow) were chosen for further analysis because these lines showed high resistance levels while developing no or a weak LTN phenotype at seedling stage. n.t. = not tested.

Transformant family T1	LTN at seedling stage	resistance	full length cDNA
2	yes	moderate	n.t.
3	yes	moderate	n.t.
4	yes	moderate to strong	n.t.
5	yes	moderate	+
6	no	no	n.t.
7	no	weak	n.t.
8	no	moderate to strong	+
9	no	moderate	+
10	no	moderate	+
11	weak	moderate to strong	+
12	yes	moderate to strong	+
13	no	weak	+
14	yes	moderate to strong	+
15	no	weak	+
16	no	no	-
17	no	weak	+
18	no	weak	+
19	no	weak	+
BG9	yes	strong	+



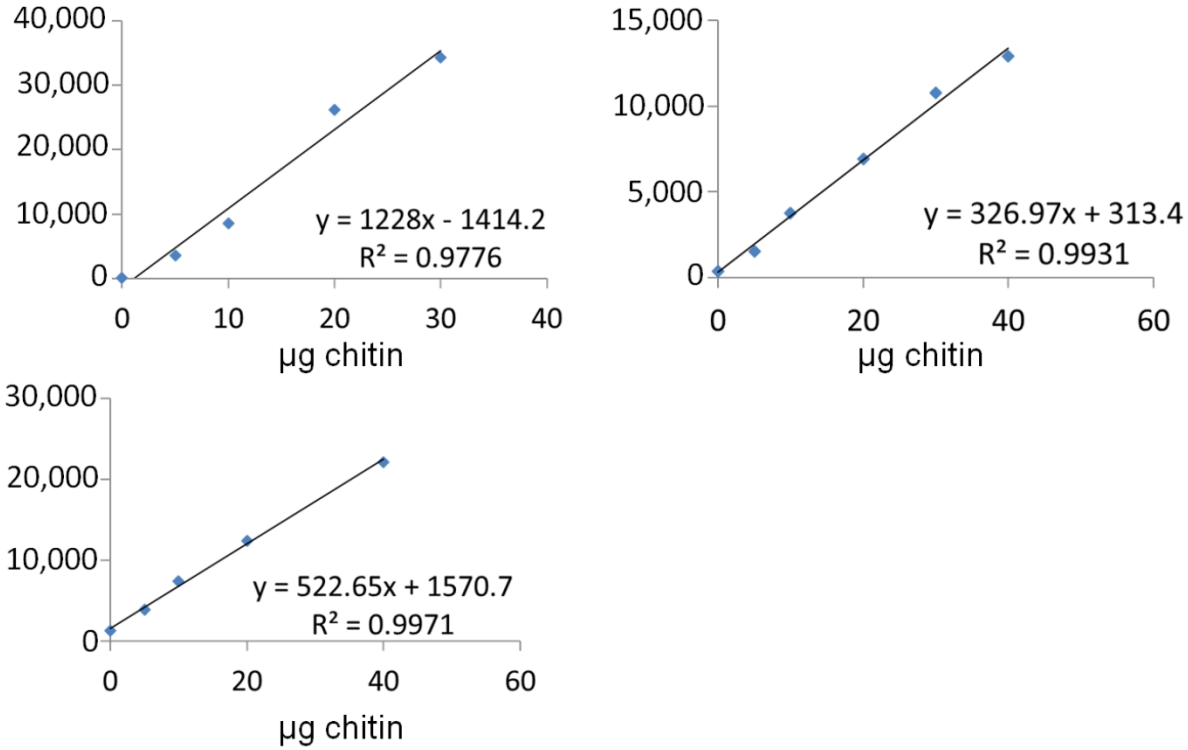
Supplemental figure 1: Southern blot of *Hv-Ger4c*::*Ta-Lr34res* barley lines.

10 µg of genomic DNA were digested with *EcoRI* and probed with a ^{32}P-labeled probe covering the *HPT* gene of the p6U vector. Black arrows show non-segregating copies, white arrows indicate missing bands, indicating segregating copies.

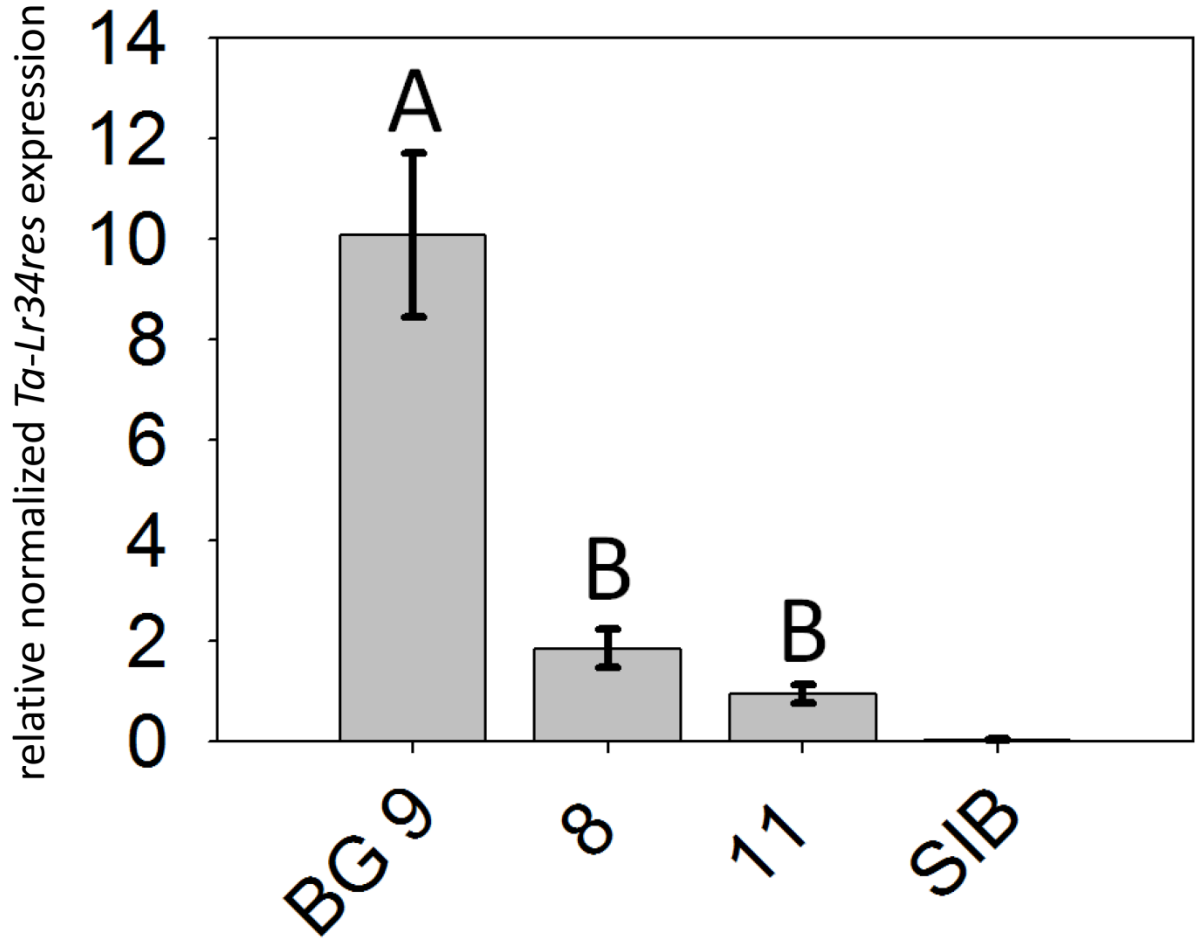


Supplemental figure 2: Additional growth parameters to Figure 2.

For assessment of growth parameters, plants were grown until maturity, 10 individual plants were harvested growth parameters as indicated were determined per plant. Plants under standard glasshouse conditions were 138 days, plants under near-field conditions were 140 days old. Error bars indicate standard errors. Transformation of raw data for statistical analysis by Tukey-Kramer HSD test and relevant p-values are mentioned in Supplemental table 2. Levels not connected by the same letter are significantly different.



Supplemental figure 3: Standard curves for chitin measurements shown in Figure 1.



Supplemental figure 4: Normalized relative expression of *Ta-Lr34res* in plants grown in the convertible glasshouse under near-field conditions

Third leaves of plants at five-leaf-stage were used for RNA extraction and expression analysis. Bars show the relative *Ta-Lr34res* expression normalized to *GAPDH* as an average of 7 biological replicates. Each replicate was measured four times (technical replicates). SIB represents the average of all sister lines representing the negative control. Error bars represent standard errors. Statistical analysis was done on $\log_{10}$-performed values using the all pairs Tukey-Kramer HSD test. Levels not connected by the same letter are significantly different. $p < 0.05$.

A) Glasshouse

Trait	dryweight	tiller	seeds per tiller	seeds per plant	g seeds per plant	1000er
test	Anova	Anova	Anova	Anova	Anova	Anova
data transformation	SQRT	SQRT	no	SQRT	SQRT	no
overal p-value	$F(5,54) = 14.4$, p-value<0.0001	$F(5,54) = 20.9$, p-value<0.0001	$F(5,54) = 23.31$, p-value<0.0001	$F(5,54) = 31.3$, p-value<p-value<2e-16	$F(5,54) = 41.6$, p-value<2e-16	$F(5,54) = 62.2$, p-value<2e-16
	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value
11 sib-11	0.75	0.92	0.86	0.41	0.23	0.98
8-11	1.00	0.62	0.99	0.74	0.77	1.00
8 sib-11	1.00	0.75	0.48	0.91	0.93	0.99
BG 9-11	0.00	<.0001	<.0001	<.0001	<.0001	<.0001
BG 9 sib-11	0.91	0.51	0.00	0.47	0.03	0.00
8-11 sib	0.55	0.12	0.99	0.02	0.01	1.00
8 sib-11 sib	0.73	1.00	0.05	0.94	0.79	0.79
BG 9-11 sib	0.00	0.00	0.00	<.0001	<.0001	<.0001
BG 9 sib-11 sib	1.00	0.08	0.01	1.00	0.93	0.01
8 sib-8	1.00	0.05	0.20	0.18	0.21	0.90
BG 9-8	<.0001	<.0001	0.00	<.0001	<.0001	<.0001
BG 9 sib-8	0.77	1.00	0.00	0.03	0.00	0.01
BG 9-8 sib	0.00	0.00	<.0001	<.0001	<.0001	<.0001
BG 9 sib-8 sib	0.91	0.03	0.00	0.97	0.23	0.00
BG 9 sib-BG 9	0.00	<.0001	0.08	<.0001	<.0001	<.0001

B) Near_field

Trait	dryweight	tiller	seeds per tiller	seeds per plant	g seeds per plant	1000er
test	Anova	Anova	Anova	Anova	Anova	Anova
data transformation	no	no	no	SQRT	SQRT	no
relevant p-value	$F(5,54) = 9.3$, p-value<0.0001	$F(5,54) = 6.4$, p-value<0.0001	$F(5,54) = 4.1$, p-value=0.003	$F(5,54) = 12.19$, p-value<0.0001	$F(5,54) = 13.1$, p-value<0.0001	$F(5,54) = 6.6$, p-value<0.0001
	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value	Post-hoc p-value
11 sib-11	0.43	0.90	0.97	0.63	0.05	0.00
8-11	0.00	0.00	0.08	0.00	0.00	0.00
8 sib-11	0.00	0.00	0.01	0.00	0.00	0.01
BG 9-11	0.88	1.00	0.01	0.13	0.02	0.01
BG 9 sib-11	0.02	0.32	0.35	0.03	0.00	0.00
8-11 sib	0.04	0.07	0.36	0.00	0.01	1.00
8 sib-11 sib	0.01	0.03	0.08	0.00	0.00	0.92
BG 9-11 sib	0.97	0.97	0.10	0.92	1.00	0.91
BG 9 sib-11 sib	0.71	0.90	0.81	0.57	0.52	1.00
8 sib-8	0.99	1.00	0.97	0.97	0.99	0.99
BG 9-8	0.00	0.01	0.99	0.03	0.04	0.99
BG 9 sib-8	0.60	0.47	0.97	0.14	0.53	0.99
BG 9-8 sib	0.00	0.00	1.00	0.00	0.01	1.00
BG 9 sib-8 sib	0.27	0.32	0.66	0.02	0.22	0.85
BG 9 sib-BG 9	0.26	0.47	0.73	0.98	0.74	0.83

Supplemental table 2: P-values, transformation of raw data and critical p-values for statistical analysis.

Differences in µg chitin per mg fresh weight and differences growth parameter values per plant across lines were tested using an ANOVA. If necessary, data were square root transformed to ensure normal distribution of residuals. When the normal distribution could not be reached, Kruskal-Wallis test were used. Multiple comparison p-values were then computed using the Post-hoc Tukey-Kramer or Kruskal-Nemenyi tests. Expression values were log10-transformed to correct the exponential character of RT-qPCR. Differences in expression were tested with an ANOVA, following the same procedure as described above. All statistical analyses were performed in R v.3.2.1. Critical p-values were used for the decision for significance to create the letter codes.

Supplemental figure 5: The convertible glasshouse enables near-field growth conditions

Plants are grown in central cylinders surrounded by buffering plants. Under dry and windless conditions the roof is automatically opened (left). Furthermore, one side wall is permanently open to allow outdoor temperatures. The system is described in Romeis et al. (2007).

Chapter D: Expanding the repertoire of functional *Lr34* disease resistance genes through targeted mutagenesis

Rainer Boni, Gabriele Buchmann, Harsh Chauhan, Goetz Hensel, Stefan T. Arold, Anne Roulin, Jochen Kumlehn, Simon G. Krattinger and Beat Keller

Summary

Durable disease resistance in wheat repeatedly evolved as a result of spontaneous mutations in genes coding for membrane-localised transporter proteins. One example is *Lr34* that provides durable and partial resistance against multiple fungal pathogens. The two predominant protein variants in wheat, Lr34res and Lr34sus differ only by two amino acid polymorphisms. In transgenic barley, it was shown by the creation of artificial Lr34 variants that only one amino acid polymorphism is essential to mediate disease resistance. This deletion ΔF546 naturally occurred after wheat domestication and affected a transmembrane helix within the ABC transporter. Here, we show that the artificial, individual deletion of the neighbouring amino acids I545 and M547, respectively had the same effect as the natural deletion of F546. In contrast, deletion of the amino acids F543 or F542/F543 did not result in enhanced disease resistance. Transgenic barley expressing the artificial HA-tagged alleles *HALr34res*, *HAΔI545* and *HAΔM547* showed increased resistance against barley powdery mildew as well as leaf tip necrosis, the characteristic senescence-like phenotype conferred by *Lr34res*. Barley lines expressing the *HALr34sus, HAΔF543* or *HAΔF542/ΔF543* allele on the other hand were susceptible to barley powdery mildew and did not develop leaf tip necrosis. Disease resistance correlated with the amount of Lr34 protein. While HALr34res, HAΔI545 and HAΔM547 proteins were detectable on a Western Blot, HALr34sus, HAΔF543 and HAΔF542/ΔF543 proteins were undetectable, even in barley lines showing comparable transcript levels as the resistant alleles. These results show that only one of several possible resistant *Lr34* variants evolved naturally. This has important implications for the engineering of novel gene variants that confer durable disease resistance, for example through genome editing. In addition, our results suggest that the difference between Lr34res and Lr34sus function is a result of the amount of protein and is not based on a change of substrate specificity.

1. Introduction

Durable, quantitative and multi-pathogen resistance genes have been described in hexaploid wheat (*Triticum aestivum*) and include *Lr67* (=*Yr46*/*Sr55*/*Pm46*), *Lr46* (=*Yr29*/*Sr58*/*Pm39*) *Lr34* (*Yr18*/*Pm38*/*Sr57*) and *Sr2* (Mago et al., 2014). All of these genes confer partial resistance against leaf rust (*Puccinia triticina*), stripe rust (*P. striiformis* f.sp. *tritici*), stem rust (*P. graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) of wheat (Ellis et al., 2014; Spielmeyer et al., 2013). *Lr67* and *Lr34* have been cloned and have been described to encode a hexose transporter (*Lr67*) and an ATP-binding cassette (ABC) transporter (*Lr34*), respectively (Krattinger et al., 2009; Moore et al., 2015).

In the case of both *Lr34* and *Lr67*, the resistant alleles evolved as a result of spontaneous sequence changes that occurred after domestication (Krattinger et al., 2013; Moore et al., 2015). The *Lr34* gene has been successfully used in wheat disease resistance breeding since the beginning of the last century (Kolmer et al., 2008), demonstrating the durability of *Lr34*. Wheat *Lr34* is accompanied by a senescence-like leaf-tip-necrosis (LTN) phenotype under certain environmental conditions (Singh and Huerta-Espino, 1997). In the wheat gene pool, two predominant alleles of *Lr34* have been found on chromosome 7DS. According to their functionality regarding disease resistance they were called *Lr34res* (resistant) and *Lr34sus* (susceptible), respectively. Interestingly, the two protein variants of Lr34 differ only in two amino acid polymorphisms, i.e. the deletion of phenylalanine at residue 546 (ΔF546) and a conversion of a tyrosine to histidine at residue 634 (T634H) in Lr34sus resulted in Lr34res. ΔF546 is caused by the deletion of a TCC in exon 11 whereas T634H is encoded by a single nucleotide polymorphism (SNP) in exon 12. In hexaploid wheat, two homoeologs were found on chromosomes 4A and 7A, respectively. Orthologous *Lr34* genes have been found in rice and *Sorghum* but not in maize, barley and *Brachypodium*. However, all homoeologs in wheat and all orthologs in other grass species showed the susceptible haplotype regarding the two critical polymorphisms (Krattinger et al., 2011). *Lr34res* was functionally transferred to barley (Risk et al.,

2013), rice (Krattinger et al., 2016), maize (Sucher et al., 2017), durum wheat (Rinaldo et al., 2016) and *Sorghum bicolor* (Schnippenkoetter et al., 2017). In barley, the two amino acid polymorphisms were further investigated. Artificial alleles of *Lr34* were created and stably transformed into barley. One of the artificial alleles, called *M1* had the deletion of the codon for F546 (as in the resistant allele) but encoded a tyrosine in exon 12 (as in the susceptible allele). The other allele, named *M2* contained no deletion in residue F546 (as in the susceptible allele) and encoded for H634 (as in the resistant allele). To test if the artificial alleles mediate resistance, barley leaf rust infection assays were performed. Whereas *M2* was susceptible to barley leaf rust, *M1* was resistant, indicating that the deletion of the codon for F546 is critical and sufficient for the resistant phenotype (Chauhan et al., 2015). The *M1* allele was also tested in wheat and showed resistance against leaf rust, indicating that the *M1* allele is able to phenocopy *Lr34res* (Chauhan et al., 2015). In this study, we analysed if additional artificial *Lr34* alleles are able to mediate disease resistance. Specifically, it was tested if the deletion of F546 is the only possibility to convert a susceptible Lr34 variant into an Lr34 variant mediating disease resistance. To do so, the *M2* allele, encoding for F546, was taken as template for site-directed mutagenesis. Four different alleles *HAΔF543*, *HAΔF542/ΔF543*, *HAΔI545* and *HAΔM547* were created. Surprisingly, *HAΔI545* and *HAΔM547* mediated disease resistance, indicating that not all possible mutations in *Lr34sus* leading to *Lr34res* have occurred naturally. As a novelty in Lr34 investigations, we included an N-terminal hemagglutinin (HA)-tag to all our artificial Lr34 proteins as well as to Lr34res and Lr34sus to allow experiments on the protein level.

2. Results

2.1 Development of barley lines transgenic for artificial *Lr34* alleles

We wanted to test if single amino acid deletions next to F546 are also able to convert a susceptible Lr34 variant into a resistant one. Based on the susceptible allele *M2,* using site-directed mutagenesis, we deleted the amino acids neighbouring F546 resulting in the artificial alleles *HAΔI545* and *HAΔM547* (Figure 1). Further, we wanted to find out if a deletion of one or two alternative phenylalanine(s) in the region of F546 would result in a resistant Lr34 variant. Therefore, we deleted one or two phenylalanine(s) next to F546 resulting in the alleles *HAΔF543* and *HAΔF542/ΔF543* (Figure 1). To allow investigations at the protein level, all alleles including *Lr34res* and *Lr34sus* were N-terminally tagged with an HA-tag. To characterize the phenotypes of the different alleles, we stably transformed barley cv. Golden Promise plants with the p6Uyeast vector containing the different *Lr34* alleles (Figure 1) under the control of the native wheat promoter. We obtained six to eighteen T0 plants of independent transformation events (later called lines) for each allele. T0 plants were grown to maturity for propagation and T1 plants were used for analysis. Plants were first tested for the presence of the *Lr34* transgene variants (alleles) by PCR on genomic DNA using a primer pair amplifying a specific *Lr34* sequence common to all *Lr34* alleles transformed.

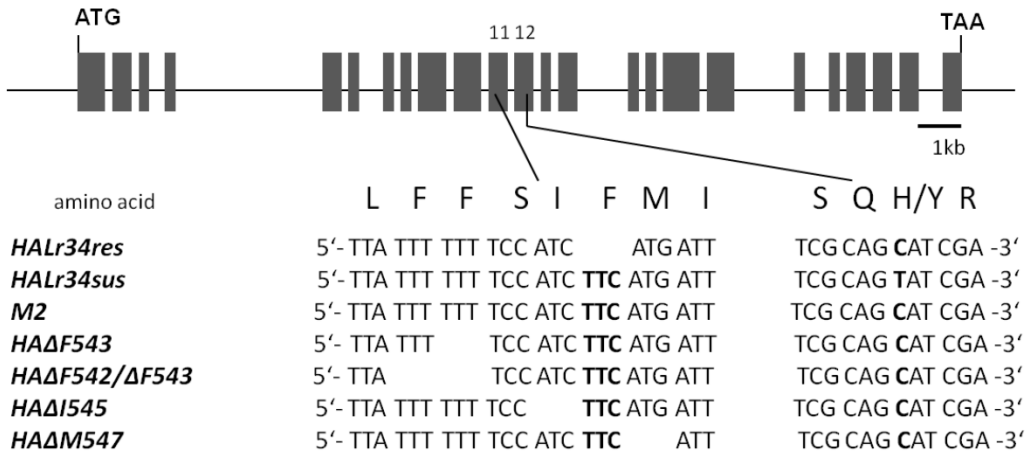


Figure 1: Generation of new *Lr34* alleles.

Lr34res, *Lr34sus*, *M2* and the newly created alleles are schematically represented. Exons are represented by boxes and introns are indicated by adjoining lines. The three critical codon changes created by site-directed mutagenesis on *M2* are given as nucleotide sequence segments.

As a next step, the highest-expressing lines per allele were determined by RT-qPCR (Figure 2A, Supplemental figure 1). The two (in the case of *HALr34sus* three) highest expressing lines for each allele were tested and confirmed for the presence of full-length cDNA by PCR amplification and used for further investigation.

2.2 The artificial *Lr34* alleles *HAΔI545, HAΔM547* and *HALr34res* mediate LTN which is expression dependant

To reveal if artificial alleles lead to a similar phenotype as mediated by *Lr34res*, 22-day-old T1 plants were checked for the development of the LTN phenotypes. *HAΔI545, HAΔM547* and *HALr34res* showed LTN of similar intensities as the *Lr34res* control line BG 9 (described in Risk et al. 2013) whereas *HAΔF543*, *HAΔF542/ΔF543* and *HALr34sus* developed no LTN phenotype (Figure 2B). Expression analysis showed a correlation of LTN phenotype with *Lr34* expression levels in lines expressing *HALr34res*, *HAΔI545* and *HAΔM547*. Only lines with *Lr34* expression above a certain threshold showed LTN (Supplemental figure 1). However, no correlation of LTN with *Lr34* expression levels were observed in barley lines expressing *HAΔF543* and *HAΔF542/ΔF543* that did not show LTN at any expression level even if expression levels were 1.5x higher *(HAΔF542/ΔF543*) compared to BG

9 (Supplemental figure 1). Interestingly, the different alleles show different expression thresholds for the LTN phenotype. In the line expressing *HALr34res*, expression levels of about 10% compared to the control line BG 9 were sufficient to develop LTN whereas expression levels about 20% and 60% compared to BG 9 were needed for LTN in lines expressing *HAΔM547* and *HAΔI545,* respectively (Supplemental figure 1). Together with the fact that even a 10x lower expression level (*HALr34res*) compared to BG 9 is sufficient for the development of LTN, we conclude that surprisingly *HAΔI545, HAΔM547* and *HALr34res* represent novel artificial alleles that are functional regarding the development of LTN phenotype (Figure 2A/B, Supplemental figure 1).

2.3 The artificial alleles *HAΔI545, HAΔM547* and *HALr34res* mediate disease resistance

We hypothesised that alleles mediating LTN (*HAΔI545*, *HAΔM547*, and *HALr34res*) confer disease resistance while alleles not leading to LTN (*HAΔF543*, *HAΔF542/ΔF543* and *HALr34sus*) do not. Therefore, infection tests were performed in T2 progeny of the selected lines except for *HALr34sus* for which T1 plants were used. Transgenic plants were infected 14 days post germination (dpg) with the barley powdery mildew isolate K1 and analysed 7 days post inoculation (dpi). Fourth leaves of infected plants were used for the macroscopic determination of pathogen symptoms. Plants expressing the alleles *HAΔI545*, *ΔM547*, *HALr34res* and *Lr34res* (BG 9) showed only a few chlorotic spots, indicating pathogen resistance (Figure 2C). Mildew colonies developed on plants transgenic for *HAΔF543*, *HAΔF542/ΔF543* and *HALr34sus* as well as on the non-transgenic sister lines (SIB), indicating full susceptibility. To quantify resistance mediated by the different alleles, the major fungal cell wall component chitin was measured using the biochemical WGA-FITC method (Ayliffe et al., 2014; Ayliffe et al., 2013). Chitin measurement was performed on 22-day-old plants 7 dpi. *HAΔI545, HAΔM547* and *HALr34res* showed similar chitin amounts as BG 9, indicating disease resistance. *HAΔF543*, *HAΔF542/ΔF543* and *HALr34sus* showed up to 11.8-fold higher chitin levels than BG 9 and were not different from values reached by SIB (Supplemental figure 2). The results of the infection assays clearly showed the functionality of *HAΔI545, HAΔM547* and *HALr34res* regarding

pathogen resistance. Further, the results demonstrate that the deletion of TTC in *Lr34sus* leading to the deletion of F546 in LR34sus, is not a unique nucleotide polymorphism leading to pathogen resistance. The same effect is achieved by the deletion of the neighbouring amino acid upstream and downstream of F546, respectively. Further, the results demonstrate that the deletions in HAΔF543, HAΔF542/ΔF543 do not alter the susceptible phenotype. The results obtained from HALr34res and HALr34sus do not differ from the control lines, demonstrating that the HA-tag does not interfere with Lr34res-mediated disease resistance.

2.4 The Lr34 variants HAΔI545, HAΔM547 and HALr34res are detectable on protein level

We wanted to test if the different phenotypes between the different Lr34 variants could be explained by variation in protein levels. Therefore, fourth to sixth leaves of 21-day-old single plants transgenic for the different alleles were pooled and used for total protein extraction, followed by microsome extraction using ultracentrifugation. Protein detection was done by SDS-Page and Western Blot. For each allele, progeny of at least two different lines (transformation events) were used. Lr34 protein was found in lines expressing *HAΔI545*, *HAΔM547* and *HALr34res*. In lines expressing *HAΔF543*, *HAΔF542/ΔF543* and *HALr34sus*, no Lr34 could be detected by Western Blot (Figure 2D), even if these lines had similar expression levels as lines expressing the resistant alleles. These results indicate a correlation of Lr34 protein amounts with LTN and disease resistance phenotype. These data also demonstrate that the main difference between Lr34sus and Lr34res function is based on different protein amounts which itself must be caused by polymorphisms in the region of F546.

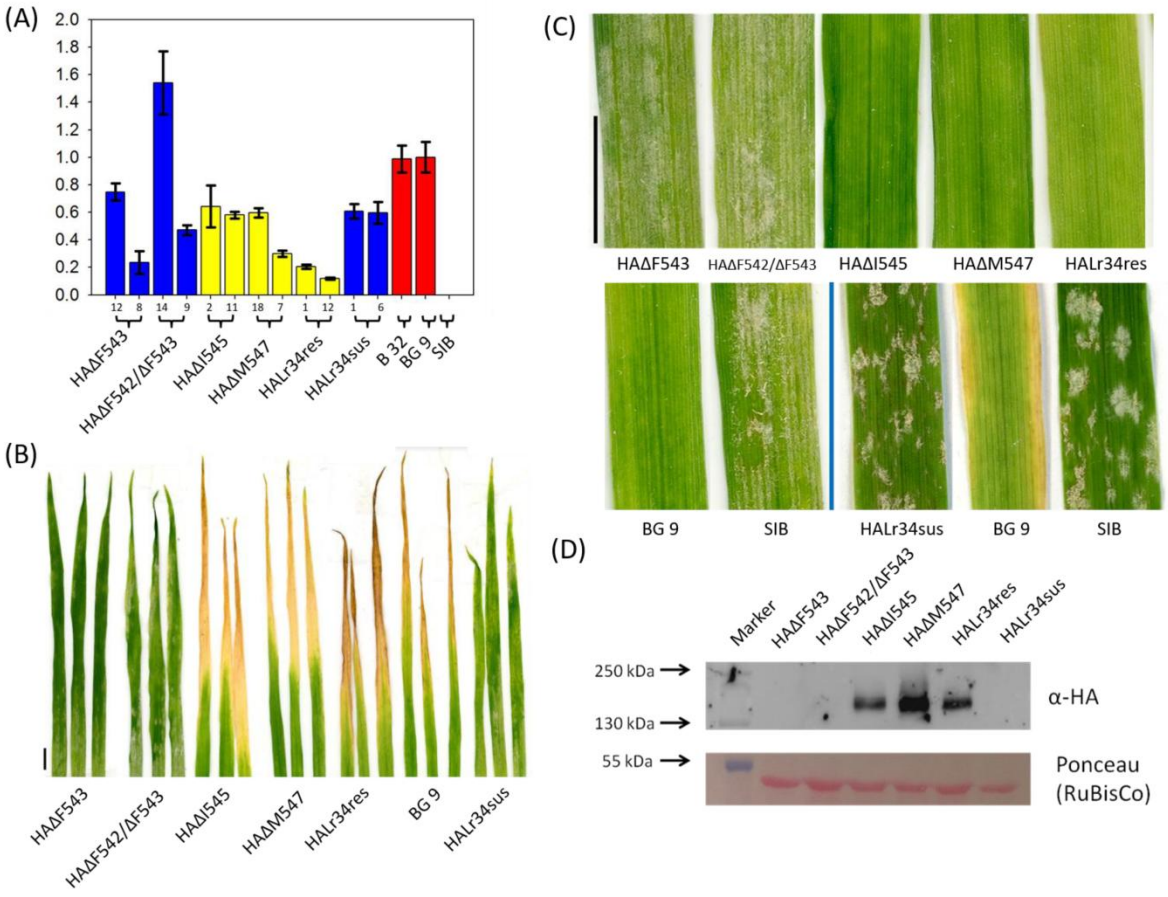


Figure 2: Amino acid deletions beside F546 lead to Lr34res phenotype.

(A) Eight second leaves of 14-day-old T1 plants were pooled within transformation events (lines) for RNA extraction end expression measurement. Expression values were normalized to *GAPDH* and set in relation to the control line BG 9. B32 expressing *Lr34res* under the native wheat promoter (Risk et al., 2013) was used as a additional control line to BG9 for the determination of LTN and the comparison of expression levels between the different *Lr34* alleles (red columns). Yellow columns indicate plants that showed an LTN phenotype, whereas blue columns indicate that no LTN was visible. Numbers below columns indicate names of the transformation events (lines). SIB represents a pool of non-transgenic sister lines that was used as negative control. Error bars represent standard error on three technical replicates. (B) The photograph shows the fourth leaves of 21-day old plants representative for each allele. The bar indicates 1cm. (C) The photograph shows the fourth leaves, representative for each *Lr34* allele, of 21-day-old plants 7 dpi with barley powdery mildew. The bar indicates 1cm. The blue bar separates pictures from *HALr34sus*, BG 9 and SIB 9 from the other lines because these lines were tested in another infection assay replicate. (D) Total leaf protein extracts were generated from pools of fourth to sixth leaves of 21-day-old plants and analyzed by Western Blot. The photograph shows Western Blot analyses of microsome extracts generated by ultracentrifugation of total protein extracts. Lr34 proteins were N-terminally tagged with a hemagglutinin (HA) epitope tag and detected using a corresponding antibody (α-HA, upper panel). Protein size is in accordance with the predicted size of Lr34 (160 kDa). Equal amounts of loaded protein extracts were checked by Ponceau staining (lower panel).

2.5 Protein structure modelling of Lr34sus

To determine the possible effect of the single amino acid deletions, we modelled the protein structure of Lr34sus based on the human sterol transporter ABCG5/ABCG8, which shows 22% amino acid sequence identity with Lr34sus. The heteromer ABCG5/ABCG8 is currently the only crystal structure of an ABCG transporter available (Lee et al., 2016). The model supports the prediction that F546 is located in the transmembrane domain 2 (Krattinger et al., 2009; 2011) (Figure 3A). According to the model, F546 is part of a helix that lines the translocation path formed by the Lr34 ABC transporter protein (Figure 3B). The importance of this helix for correct translocation is illustrated in the human ABCG5 by a mutation of a close-by arginine (R419H) of this helix, which leads to sitosterolaemia, an autosomal disorder that is caused by an increased transport of sterols into the bile and gut lumen (Lu et al., 2001) (Figure 3B). The transmembrane helix forms a kink in the region of F546 at S544 (Figure 3B). Interestingly, all deletions in Lr34sus (HAΔI545, ΔF546 and ΔM547) leading to disease resistance are located below this kink (towards the intracellular membrane layer), whereas all deletions not altering the susceptible phenotype (HAΔF543, HAΔF542/ΔF543) are located above the kink (towards the extracellular membrane layer). Thus, the model supports that deletion of the more internal residues I545, F546 and M547 leads to more severe changes in protein folding, and hence in protein stability and translocation activity. Y634 is also located in the transmembrane domain 2, however further towards the intracellular space. Modelling supports that its substitution with a histidine does not introduce significant steric hindrance or structural alterations, in agreement with our experimental observations (Figure 3C).

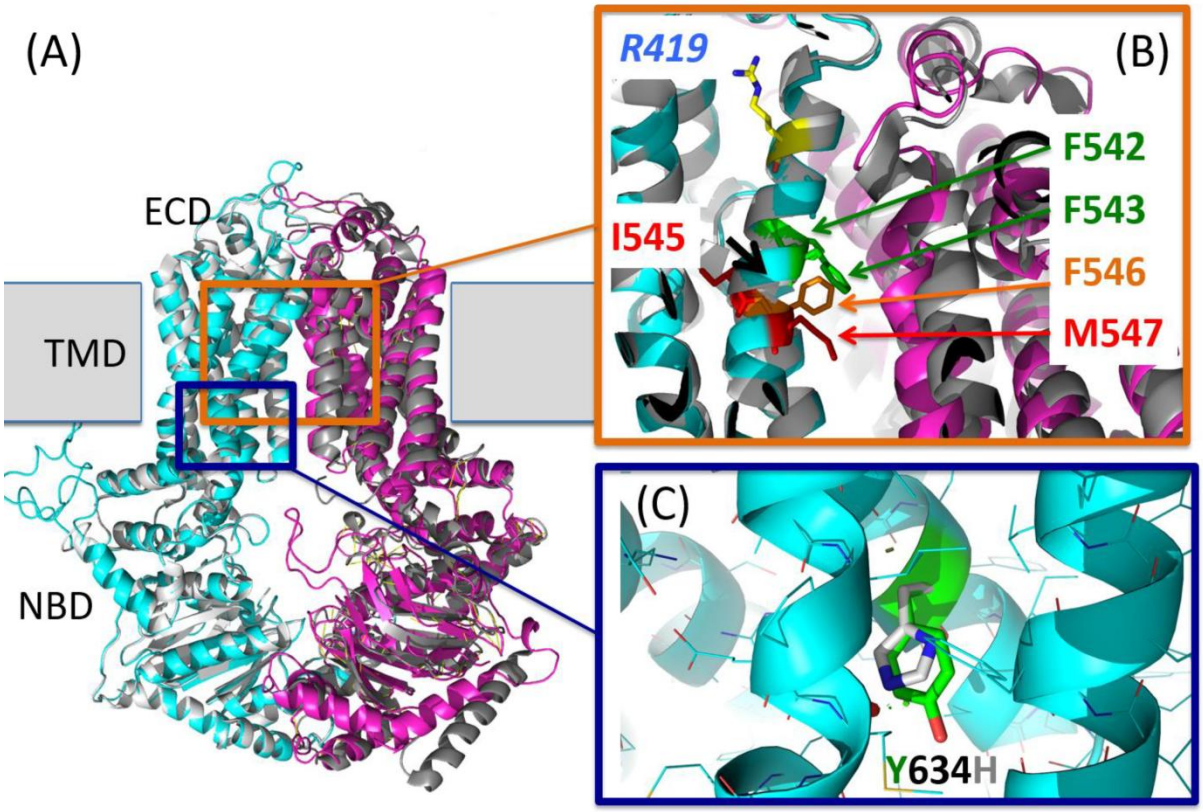


Figure 3: *In silico* structural analysis of Lr34sus.

(A) The 3D structure of Lr34sus was modeled based on the crystal structure of the human ABCG5 (light gray) and the ABCG8 (dark grey) heterodimer as template (PDB 5do7). Cyan and magenta represent Lr34 N- and C-terminal transporter domains, respectively. ECD: Extracellular domain, TMD: Transmembrane domain, NBD: Nucleotide binding domain. Grey boxes represent membrane layers. (B) Magnification of orange box from (A) highlighting F546, amino acids of the artificial Lr34 variants, and the sitosterolemia causing mutation ABCG5-R419(H). A black arrowhead indicates the kink in the transmembrane helix. (C) Magnification of the orange-boxed region of (A) showing the location of Y634 (carbons are in green in the stick representation) and its substitution by a histidine (grey).

3. Discussion

3.1 Sequence-guided mutagenesis expands the repertoire beyond natural resistant *Lr34* alleles

In wheat, the different phenotypes of Lr34res and Lr34sus are based on the two single amino acid polymorphisms ΔF546 and Y645H (Krattinger et al., 2011). Chauhan et. al (2015) have shown in barley and wheat that the deletion of F546 is sufficient and necessary to convert the Lr34sus protein into a resistance-mediating version. Here, we show that additional deletions, namely ΔI545 or

ΔM546, lead to similar phenotypes as mediated by Lr34res whereas deletions of F543 and deletions of F542/F543 were indistinguishable from the susceptible phenotype. Krattinger et al., (2011) identified three different *Lr34* homoeologs in wheat referred as to *Lr34-A*, *Lr34-B*, and *Lr34-D* according their chromosomal localisation. *Lr34-D* mostly occurs as one of two alleles *Lr34res* and *Lr34sus*. *Lr34-A* was interrupted by the insertion of several repetitive elements and was not expressed. *Lr34-B* was shown to be expressed and the amino acid identity of Lr34-B is 97% to Lr34-D. *Lr34-B* was detected in all wheat cultivars. Orthologs were found in rice and *Sorghum*. All *Lr34-B* homoeologs and *Lr34* orthologs in other species showed the susceptible haplotype (Krattinger et al., 2011). This indicates that different variants of *Lr34* evolved naturally, but interestingly none of them led to disease resistance. Our results demonstrate that not all possible resistance-conferring *Lr34* variants evolved naturally. This might be explained by the fact that *Lr34res* evolved after domestication about 10,000 years ago, a short time period in evolutionary scale. Perhaps, alternative mutations occurred naturally before domestication but disappeared within a few generations because of negative pleiotropic effects, which were not compensated by breeding-based selection as it was done for *Lr34res*.

The use of *Lr34res* in high-input crop growing systems was recently shown to lead to yield penalties in wheat. Johnston et al. (2017) compared different *Lr34res* and *Lr34sus* lines regarding yield in New Zealand. Further, it was tested how yield is affected with or without of fungicide control. *Lr34res* is advantageous compared to *Lr34sus* regarding enhanced disease resistance and therefore, lines with the *Lr34res* allele have higher yield if no fungicides are applied (Johnston et al., 2017; Singh and Huerta-Espino, 1997). In contrast, with high fungicide use, average yield penalties of 4.4% in *Lr34res* lines were observed compared to *Lr34sus* lines. (Johnston et al., 2017; Singh and Huerta-Espino, 1997). Although our artificial alleles show strong LTN in barley, in wheat they might show less yield penalties under fungicide application compared to *Lr34res* lines. To test this, one could introduce the mutations ΔI545 and ΔM547, respectively in *Lr34sus* lines by genome editing. The resulting wheat

lines expressing *ΔI545* and *ΔM547*, respectively could be tested in field trials analogously to the work of Johnston et al. 2017.

3.2 Both Lr34 and Lr67 differ by single amino acid polymorphisms that result in a common LTN phenotype

Interestingly, similar to *Lr34res/sus*, *Lr67* has also two alleles referred to as *Lr67res* and *Lr67sus*. And *Lr67res* is also accompanied by the LTN phenotype. *Lr67res* evolved from *Lr67sus* by two nonsynonymous SNPs in exon 2 and 3 resulting in R144G and L387V. Glucose uptake experiments in yeast have shown that *Lr67res* is inhibited in glucose uptake compared to *Lr67sus* and that the SNP in exon 2 but not in exon 3 was crucial for the phenotypic change (Moore et al., 2015). The example of *Lr67res* represents a gain-of-function mutation event regarding disease resistance but was impaired in its functionality (glucose import). However, loss of function mutants were shown to be more susceptible to wheat powdery mildew (Moore et al., 2015). Both Lr67 and Lr34, the differences between the resistant and the susceptible allele seem to be related to a dosage dependant effect. In the case of Lr67 this effect is related to the glucose uptake efficiency whereas in Lr34 the effect is based on protein amounts. Together we conclude, that both, Lr67res and Lr34res are involved in a common (still unknown) mechanism at different positions in the metabolic network and contribute by finetuning to similar phenotypes in disease resistance and LTN.

3.3 Single amino acid polymorphism can drastically alter protein function

Our results demonstrate that in addition to the naturally existing single amino acid polymorphisms within Lr34 (Krattinger et al., 2009), artificially induced changes at the single amino acid level lead to changes in protein function. Protein function can be changed by altered protein interactions (substrate specifity and efficiency in the case of transporters) or protein stability. An example for an altered protein-protein interaction is the deletion of phenylalanine F508 of the anion channel named cystis fibrosis transmembrane conductance regulator (CFTR). There, deletion ΔF508 leads to a non fully glycosylated anion channel which leads to minimal activity in bronchial epithelial cells of human

patients with cystic fibrosis (Pankow et al., 2015). Interestingly, CFTR is an ABC transporter (Guggino and Stanton, 2006) and a phenylalanine is affected. However, protein sequence (https://www.ncbi.nlm.nih.gov/protein/NP_000483) analysis revealed that ΔF508 is located in NBD1 (http://wlab.ethz.ch/protter). Therefore, ΔF508 in CFTR and the deletions in the Lr34 variants are not directly comparable.

In order to check if the difference of resistant and susceptible Lr34 alleles is based on protein level, all artificial alleles as well as *Lr34res* and *Lr34sus* were designed with a N-terminal HA-tag to enable protein detection using SDS-Page and Western Blot. Doing so, we detected the proteins of HALr34res, HAΔI545 and HAΔM547 but not HALr34sus, HAΔF543 and HAΔF542/ΔF543. The increased protein amounts of HALr34res, HAΔI545 and HAΔM547 could not be explained by mRNA silencing of the susceptible alleles due to the fact that all alleles were expressed at high levels and for all constructs intact full-length cDNA was detected.

There are different scenarios why resistant Lr34 variants showed accumulated protein levels compared to susceptible proteins. It is possible that the protein sequence in the region of ΔF546 is crucial for correct protein folding, localisation, stability or function. HALr34res, HAΔI545 and HAΔM547 do not share the amino acid sequence "SIFM" with HALr34sus, HAΔF543 and HAΔF542/ΔF543 (Figure 1). "SIFM" could lead to protein misfolding detected in the endoplasmatic reticulum (ER) followed by Endoplasmatic Reticulum Associated protein Degradation (ERAD) (Huttner and Strasser, 2012; Meusser et al., 2005). *Arabidopsis* TURAN (TUN) is a protein in the ER involved in N-glycosylation of ANX1 a protein involved in pollen tube reception. ANX1-GFP fluorescence disappeared in *tun* mutants but was recovered by two different ERAD inhibitors, indicating that non N-glycosylated ANX1 underwent ERAD (Lindner et al., 2015). Analogously, the hypothesis that HALr34sus, HAΔF543 and HAΔF542/ΔF543 undergo ERAD could be tested by incubating leaf material in the ERAD inhibitors prior to Western Blot assays, assuming that HALr34sus, HAΔF543 and HAΔF542/ΔF543 become detectable after treatment. However, the fact

that Lr34sus is highly conserved in the wheat gene pool reduces the likelyhood that susceptible Lr34 variants are misfolded and therefore it could be that the correct protein is only made in certain conditions (for example stress).

Another possibility might base on protein degradation via phosphorylation. Therefore, we were interested if the "SFIM" motive is known to be involved in protein phosphorylation. Screening for "SFIM" in databases for phosphorlyation sites on PhosPhAt 4.0 and P3DB revealed only one hit "AYQDGHSIFMELIDRGMLK" originating from an *Arabidopsis* LRR protein (AT5G45510.1) which was phosphorylated at Y and S (Engelsberger and Schulze, 2012; Nemoto et al., 2011; van Bentem et al., 2008). One the one hand the fact that one hit was found shows that our hypothesis is plausible. On the other hand, one hit alone also indicates that the "SIFM" is not a conserved motif for phosphorylation, which speaks against our hypothesis. In the case of Lr34 in we would hypothesize that the "SFIM" sequence is responsible for posttranslational degradation via phosphorylation in HALr34sus, HAΔF543 and HAΔF542/ΔF543. One idea to restore HALr34sus, HAΔF543 and HAΔF542/ΔF543 detection by Western Blot and testing this hypothesis could be done by plant material treatment with the proteasome inhibitor MG132, eventually followed by an ubiquitin detection as it was already reported (Goehre et al., 2008; Mendiondo et al., 2016). However, based on the modelling, degradation via phosphorylation seems not to be a likely explanation for the different protein amounts found in resistant and susceptible Lr34 variants. For protein phosphorylation the serine would need to be accessible to (cytoplasmic) kinases, which here is not the case because the serine is embedded in the TMH and therefore hidden in the channel within the TMD.

Recently, mis-spliced *Lr34* transcripts were described in winter wheat (Fang et al., 2017). Mis-splicing could have led to reduced Lr34sus protein amounts observed in our experiments. However, in our case, mis-splicing could be excluded as we sequenced the full-length cDNA that was complete and correct.

Recently, the existence of a Lr34 suppressor was shown by crossing different Chinese wheat landraces with Chinese Spring (containing *Lr34res*) or Avocet (containing *Lr34sus*) (Wu et al., 2015). First, several landraces were shown to be susceptible to stripe rust although they contained *Lr34res*. Second, F1 progeny of the cross between Sichuanyonggan 2 (*Lr34res*) and Chinese Spring were susceptible to stripe rust, indicating that Sichuanyonggan 2 contains an *Lr34* suppressor gene (Wu et al., 2015). The identification of the suppressor and on which level the interaction works (for example by reducing the *Lr34res* expression) remains elusive. Once the suppressor has been cloned it would be interesting to test if it is also effective against *HALr34res*, *HAΔI545* and *HAΔM547* in barley. Prior the cloning of the suppressor, Avocet and/or Chinese Spring transgenic for *HAΔI545* and *ΔM547*, respectively could be crossed with Sichuanyonggan 2 to evaluate if the suppressor also is effective against the artificial resistant alleles presented in this work.

Finally, our data show that the difference of Lr34res and Lr34sus is based on protein amounts of Lr34res and Lr34sus, respectively. The absence of protein in the Western Blot assay could be due to the absence of protein or to protein amounts below detection levels. Therefore, it is not clear if Lr34sus is totally absent or present in little amounts. To test this, more sensitive techniques as quantitative mass spectroscopy could be used.

3.4 Potential use of artificial alleles generated by biotechnological approaches for agricultural application

Our results represent a case study showing that artificially induced single amino acid polymorphisms might be useful in crop breeding. Similar approaches based on single amino acid polymorphisms have been described previously. The different approaches strongly in their complexity. Here, knock out mutations are considered as approaches with low complexity. To introduce a stop codon or a frameshift, sequence information on the gene of interest is mostly sufficient. In contrast, artificial gain-of-function mutations are considered as more complex because sophisticated knowledge on protein function is needed.

One example of low complexity is the knock-out of the three *Ta-Mlo* homoeologs *Ta-Mlo-A1*, *Ta-Mlo-B1* and *Ta-Mlo-D1* in hexaploid wheat (Acevedo-Garcia et al., 2017). *Ta-Mlo* is the ortholog of barley *Mlo*, a recessive resistance gene in barley, mediating durable disease resistance against several strains of barley powdery mildew (Buschges et al., 1997). Acevedo-Garcia et al. managed to knock out all three wheat homoeologs by single amino acid exchanges using a Targeting Induced Lesions In Genomes (TILLING) approach resulting in resistance against wheat powdery mildew. The same was also achieved by using the TALEN or CRISPR-Cas9 methods (Wang et al., 2014).

An example with high complexity is represented by the NBS-LRR protein PM3. PM3 mediates disease resistance in wheat against powdery mildew. PM3 occurs in form of several alleles leading to a hypersensitive response (HR) with different extents of race-specifity spectra (Brunner et al., 2010; Srichumpa et al., 2005). Normally, the coexpression of the matching *Avr* is needed to get *R*-gene-mediated HR in transient expression assays. A single amino acid change in the MHD motif (D502V in PM3A/B and D501V in PM3C/F) was sufficient to create autoactive PM3 versions when infiltrated in *N. benthamiana* (Stirnweis et al., 2014). Further, Stirnweis et al. (2014) showed that two amino acid changes in the ARC domain (L456P and Y458H) led to enhanced HR mediated by PM3c but not PM3f compared to PM3a/b harboring P456 and H458. However, the same mutation led to an extended resistance spectrum of *Pm3f* when expressed in wheat (Stirnweis et al., 2014). Thus, these results demonstrate that single amino acid changes based on different natural alleles might be used to enhance intensity and spectrum of disease resistance genes. However, the findings from PM3 also show that the situation is very complex even among alleles of the same gene.

Both *Lr34* and *Lr67* encode for transporter proteins that lead to the similar phenotypes as LTN and disease resistance. Therefore, we speculate that other transporter proteins might also mediate quantitative resistance. It would be interesting to test if the critical amino acid changes discovered in this work could be used to convert other transporter proteins into proteins mediating quantitative resistance. Although no ortholog of *Lr34* is present in barley, one could try to find homeologous

sequences in barley ABCG transporters and introduce the mutations ΔI545 or ΔM547. This could be done using CRISPR-Cas9 (Jinek et al., 2012; Ochiai, 2015) for which elaborated protocols exist (Liang et al., 2017; Yang, 2017).

4. Material and methods

4.1 Cloning of deletion constructs and generation of stably transformed barley

The *M2* allele (Chauhan et al., 2015) containing the three base pairs 'TCC' encoding for the phenylalanine at position 546 as in *Lr34sus* and the 'CAT' codon for tyrosine at position 634 as in *Lr34res* was chosen as sequence template. A fragment of 2,958bp covering the phenylalanine codon using the primers Lr34-1200f and Lr34-3000r (Supplemental table 1), was cloned in the pSC-Bamp/kan vector using the StrataClone Blunt PCR Cloning Kit (Agilent Technologies, USA) according to the manufacturers protocol. Deletions were introduced by performing a PCR-based site-directed mutagenesis with primers (Supplemental table 1) designed online with the QuickChange Primer Design tool (Agilent Technologies, USA) and the utilisation of the Phusion polymerase (New England Biolabs, USA). PCR products were transformed in the *E. coli* strain DH5α after *DpnI* (New England Biolabs, USA) digestion. The newly created pSC-Bamp/kan vectors containing different deletions were isolated using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA). The assembly of the whole *Lr34* alleles with the deletions was done by yeast homeologous recombination. To do so, the vector p6U was digested with *EcoRI* (NEB) and the Leu2 cassette, amplified by Primer dst236 and dst237 on the template pRS305, was introgressed to create a yeast compatible vector, called p6Uyeast. The p6Uyeast vector was linearized using *SfiI* and different PCR products based on p6U::*Lr34res* (Risk et al., 2013) and the pSC-B deletion sequences were transformed in *S. cerevisiae* strain RGSY for homeologous recombination. Taking advantage of this method, which allows assembling of different DNA fragments without interrupting sequence fragments resulting from the cloning vector, we fused an HA-tag in front of the start codon of all constructs. The p6Uyeast plasmids with the different alleles were taken for *Agrobacterium*-mediated stable transformation of

barley cv. Golden Promise as described before (Hensel et al., 2009). The protocol was slightly adapted by adding 1.25 mg/l $CuSO_4$ pentahydrate to the callus-forming media and the use of *A. tumefaciens* strain AGL-1.

4.2 Selection of transgenic lines used for analysis

Lines with the highest expression levels and showing full-length cDNA amplification were chosen for further analysis. The identification of high expressing lines was based on RT-qPCR. Second leaves of 8 T1 progeny of each independent transformation event were pooled for RNA extraction and expression level measurements. Expression values were normalized to *GAPDH* and set in relation to the highly expressing lines BG 9 containing *Lr34res* under the control of the native promoter. Experiments were performed on T1 or T2 plants as indicated. Homozygous lines could not been found for all alleles within the lines tested. In segregating lines, plants were selected based on the analysis of genomic DNA for the presence of the transgene in each experiment.

4.3 RNA extraction

Total RNA extraction was achieved with the SV Total RNA Isolation system (Promega, USA). RNA integrity was tested by electrophoresis on a 1.2% agarose gel in 1x sodium-borate buffer. After RNA concentration measurement on a Nanodrop ND-1000 spectrophotometer, 500ng RNA were taken for cDNA synthesis with the iScript^{TM} advanced cDNA synthesis kit (Bio Rad, USA).

4.4 Expression measurement using RT-qPCR

RT-qPCR was performed on a CFX96 or a CFX384 Touch Real-time PCR machine (Bio Rad, USA).

Reaction was set up by 4 technical replicates of 5µl KAPA SYBR fast qPCR mix (KAPA biosystems, USA), the primers *Lr34res*_R and RB_224 (500nM final concentration, Supplemental table 1) and 4 µl of 1:20 diluted cDNA per sample. *Lr34* expression values were normalized to the reference gene *GAPDH* (Supplemental table 1).

4.5 Full-length cDNA amplification and detection of transgenic barley

Lines selected for high expression levels of the transgenes were checked for full length cDNA amplification. In this case, cDNA was made using the M-MLV reverse transcriptase according to manufacturer's protocol (Invitrogen, USA) and a poly-T (30) oligo. Touch-down PCR (1. 98°C 3min, 2. 98°C 10sec, 3. 67°C 20sec, 72° 2min 50sec, 4. go 10x to 2. while reducing annealing temperature 1°C per cycle, 5. 95°C 10sec; 6. 55°C 20sec, 7. 72°C 2min 50sec, go 25x to 5, 8. 72° 5min) was performed using the primers FLC_F and FLC_RW (Supplemental table 1) was performed to check for the full-length amplicon of 4,221bp (*HALr34sus*) 4,216bp (*HALr34res*, *HAΔF542*, *HAΔF543* and *HA*ΔM547) and 4,319bp (HAΔF542/ΔF543), respectively. Total extraction of genomic DNA was done by the CTAB method (Stein et al., 2001). Transgene presence was checked by PCR using the Primers RB_ 46 and RB_133 (Supplemental table 1).

4.6 Growth conditions and infection assays

Sawing was done in soil-filled (Einheitserde Profi Substrat, Einheitserde Werkverband e.v., Germany) 96-well Jiffy pots. Before transferring to the growth chamber (16h, 20°C, light and 8h, 17°C, darkness), plants were incubated over night at 4°C in the dark for synchronisation of germination. For seed propagation, plants were transferred to 2l pots (one plant per pot) and grown in the glasshouse at standard growth conditions. Infection with powdery mildew was performed by shaking spores of isolate K1 from approximately 25 plants (21 dpg) of 7dpi barley cv. Golden Promise grown in one pot. Infected plants were transferred to a Sanyo MLR351 incubator (Sanyo, Japan) (16h, 20°C, light and 8h, 20°C, darkness).

4.7 Quantitative determination of chitin amount

Chitin measurement was performed as described in Ayliffe et al. (2013 and 2014) with some adaptions. Samples were not autoclaved but heated for 20 min in a steam cooker. Samples were diluted 1:10 prior measurement of 4 technical replicates with the fluorometer Synergy H1 Hybrid

311 Reader (BioTek Instruments GmbH, Switzerland). The software Gen5, version 2.03.1 (BioTek Instruments GmbH, Switzerland) was used for read-out analysis. Defined amounts of chitin from shrimp shells (Sigma-Aldrich, USA) were used to determine standard curves (Supplemental figure 2) to calculate chitin amounts.

4.8 Protein extraction, SDS-Page and Western Blot

Total protein extracts were optained by grinding leave pools of plants as indicated using a mortar and liquid nitrogen. 3ml of extraction buffer (100mM NaCl, 50mM Tris/HCl pH8, 25mM sucrose, 5mM EDTA, 10% glycerol, 1% Triton X-100, 5mM DTT and one tablet of cOmplete Tablets, EDTA-free, EASYpack protease inhibitor cocktail (Roche, Switzerland) per 10ml extraction buffer) was added per 1g grinded leaf material. Soluble proteins were separated from debris by two centrifugation steps at 15,000g for 20 minutes and 10 minutes, respectively. Enrichement of the membrane fraction was achieved by ultracentrifugation (100,000 g) using a Optima Xpn 100 ultracentrifuge (Beckman Coulter, USA). Protein concentration measurement was achieved by Bradford assay using Protein Assay Dye Reagent Concentrate (BIO-RAD, USA) and a Spectra Max 190 spectrometer (Bucher biotec, Switzerland). Equal protein amounts (24µg) were separated on a 6.5% Acrylamide/Bisacryl gel (BIO-RAD, USA) and blotted to a nitrocellulose membrane (Amersham™ Protran™ 0.2µm NC, GE Healthcare life sciences, USA) using the Mini-Protean II system (BIO-RAD, USA). Blots were incubated with 1:1,000 rat monoclonal antibody (Anti-HA-Peroxidase High Affinity (Roche, USA). Signals were detected using the WesternBright™ Quantum kit (Advansta, USA) and quantified with a Fusion FX6-XT-820.EPI camera and the EvolutionCap software (Vilber Lourmat/Witec AG, Switzerland).

4.9 Statistical analysis

Differences in µg chitin per mg fresh weight across lines were tested using an ANOVA. If necessary, data were square root transformed to ensure normal distribution of residuals. Multiple comparison p-values were then computed using the Post-hoc Tukey-Kramer test.

4.10 Computational modelling

SwissModel (Arnold et al., 2006) was used to produce homeology models. The structure of the phosphatase domain was modelled based on the human sterol transporter ABCG5/ABCG8, that showed 22% amino acid sequence identity with Lr34sus (Lee et al., 2016, Protein Data Band (PDB) accession 5do7). 3D structures were manually inspected, and mutations evaluated, using the Pymol program (pymol.org).

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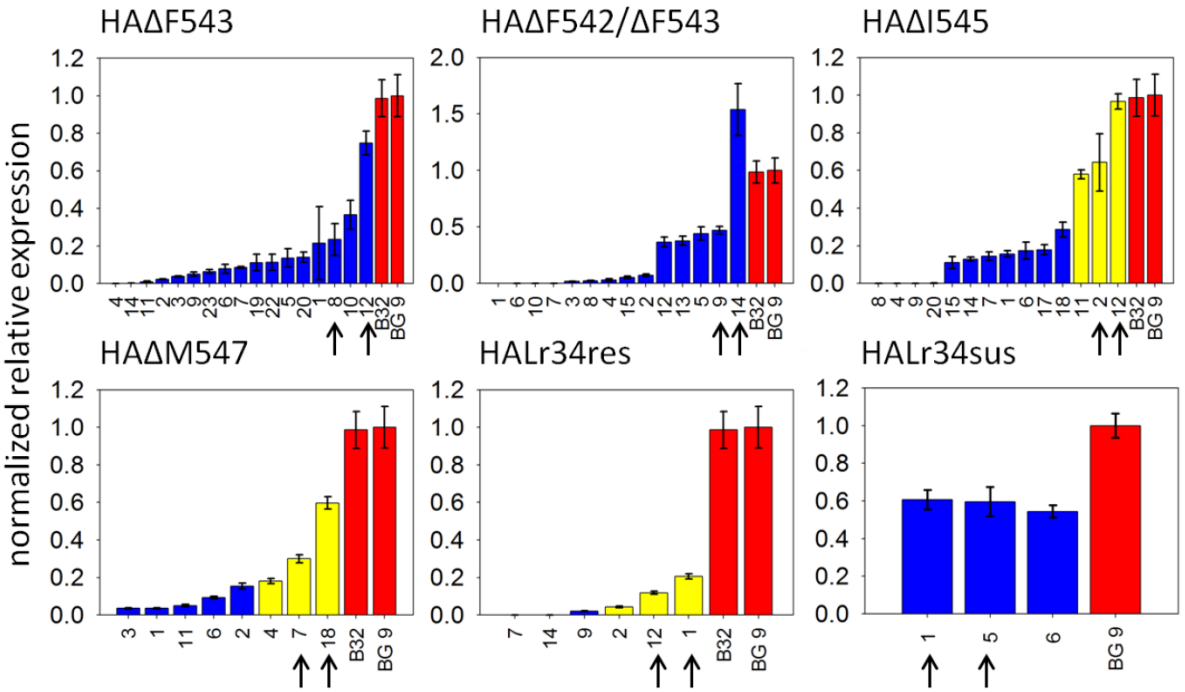
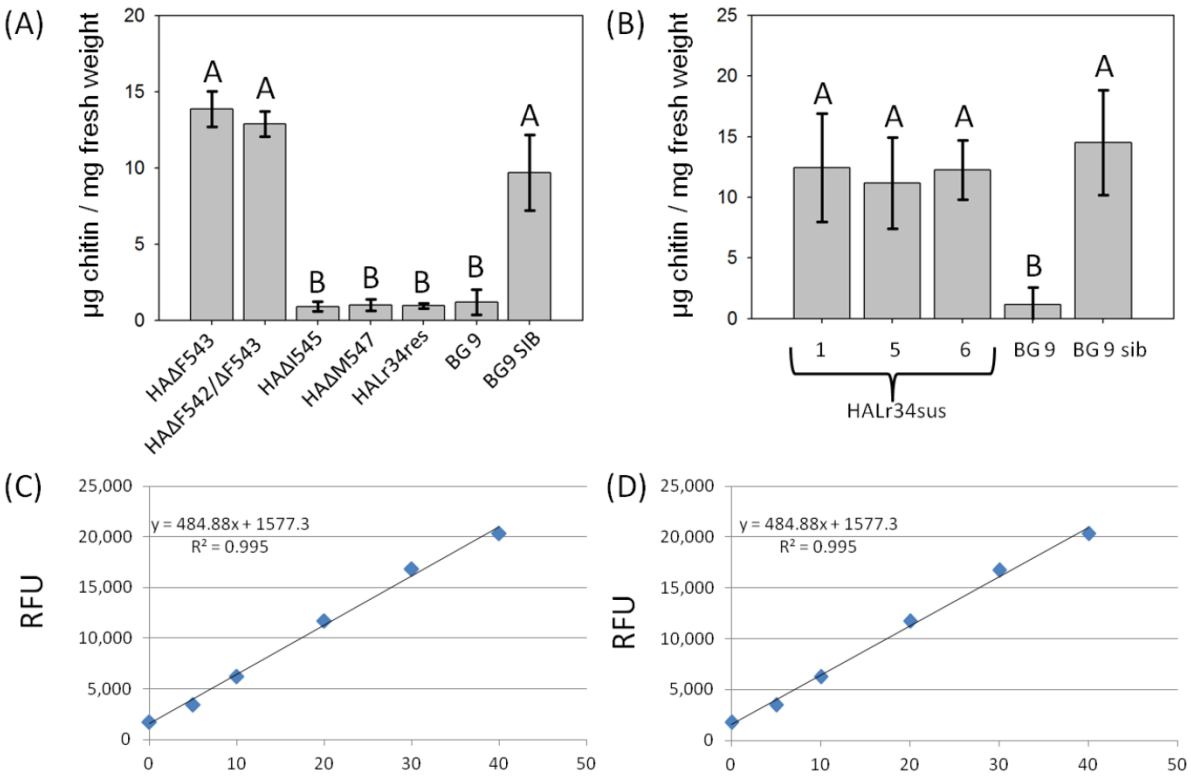
7. Supplemental material



Supplemental figure 1: Expression analysis reveals sequence- and expression-level-dependant phenotypes.

Second leaves of eight 14-day old T1 progeny of each independent transformation event were pooled for RNA extraction and expression level measurements. Expression levels were normalized to *GAPDH* and set in relation to the high expressing *Lr34res* line BG 9. Columns colored in yellow and red indicate LTN in transgenic families and control lines, respectively. Blue columns indicate no LTN. Error bars represent standard error of three technical replicates. T2 progeny of lines marked with an arrow were used for infection experiments.



Supplemental figure 2: Chitin measurements confirm disease resistance.

(A): Fourth leaves of 21-day-old plants 7dpi were taken for chitin measurement. Three to four biological replicates of the two highest expressing transformation events (lines) were measured. Chitin measurements for the Bars show mean values as amount of chitin in µg chitin per mg fresh weight. Error bars indicate standard error. Statistical analysis was done non-transformed values by performing an all pairs Tukey-Kramer test. Levels not connected by same letter are significantly different. P-value: < 0.01. (B): Separate assay, as HALr34sus was tested later. Fourth leaves of 21-day-old plants 7dpi were taken for chitin measurement. Three to four biological replicates of the three highest expressing transformation events (lines) were measured. Chitin measurements for the Bars show mean values as amount of chitin in µg chitin per mg fresh weight. Error bars indicate standard error. Statistical analysis was done non-transformed values by performing an all pairs Tukey-Kramer test. Levels not connected by same letter are significantly different. P-value: < 0.01. (C) standard curve for (A). (D): standard curve for (B).

Supplemental table 1: Primer sequences.

Sequences of primers used for cloning, Full-length cDNA amplification, genotyping and RT-qPCR, respectively.

Name	Sequence 5' to 3'	purpose			Reference
L34-1200f	TTGAGGAGTGTGGGTTCATATGCC	cloning			this work
Lr34-3000r	TTGACTGCACGAATAACAATGGCTG	cloning			this work
dst236	CTCCACGAAAATATCCGAACGCAGCAAGATTGGGTCCTTTTCATCACGTGC	cloning			this work
dst237	TGCCCAGGCAAGACCGAGATGCACCGCGATGCGGCCGCCACCGCGGT	cloning			this work
dst196	CCAATATATCCTGTCAAACACTG	cloning			this work
dst239	CAGCATAATCTGGAACATCGTATGGATACATCTCAAGATGGGTGAGTTAAATCG	cloning	HA-Tag		this work
dst238	ATGTATCCATACGATGTTCCAGATTATGCTGAGGGCCTCGCAAGAGAGAC	cloning	HA-Tag		this work
Lr34-1200r2	GGCATATGAACCCACACTCCTCAA	cloning			this work
Lr34-3000F2	GCAGCCATTGTTATTCGTGCAGTC	cloning			this work
Lr34-3600r	TTGACTGCACGAATAACAATGGCTG	cloning			this work
Lr343600F2	CACCATAGGCGCTTATAATGATCAGAC	cloning			this work
dst167	ATGCAAGCTGATCCACTAG	cloning			this work
FLC_F	GAGTACGGCTAGGCAATAGC	full length cDNA amplification			this work
FLC_R	GGCAAGTAGCTATATCTGTAAC	full length cDNA amplification			this work
RB_46	ACATTCCCCACAGCTTAC	genotyping			this work
RB_133	CTGATGGATTGTACAGACTACT	genotyping			this work
sqRTF-HvGAPDH	CCGGGTTCCCACTGTGGAT	RT-qPCR	primer efficiency	E=102.0% R^2=0.977 Slope=3.466 y-int=21.012	this work
sqRTR-HvGAPDH	TGACTAGCAACTCGGTGCGG	RT-qPCR	primer efficiency	E=102.0% R^2=0.977 Slope=3.466 y-int=21.012	this work
Lr34res_R	ACTGGCAGAAGAACCTTGAAACA	RT-qPCR	primer efficiency	E=101.7% R^2=0.977 Slope=3.282 y-int=25.000	Risk et al. 2013
RB_224	ACTGGATTCGCTTGTATGGATA	RT-qPCR	primer efficiency	E=101.7% R^2=0.977 Slope=3.282 y-int=25.000	this work

Chapter E: Conclusions and outlook

1. Conclusions

1.1 Similarities of *Lr34* and *Lr67* indicate that quantitative disease resistance is achieved by distinct fine-tuning of signalling pathways involved in a common resistance mechanism

Both Lr34res and Lr67res evolved from single amino acid polymorphisms in their susceptible variants (Krattinger et al., 2009; Moore et al., 2015). Lr67res was shown to be impaired in glucose transport while the substrate of Lr34res is still not known. Both genes lead to similar phenotypes such as LTN and quantitative disease resistance (Spielmeyer et al., 2013). *Lr34res* mediates up-regulation of pathways involved in basal and induced disease resistance (Chapter B). Because of the similar phenotypes, we assume that *Lr67res* mediates the upregulation of similar pathways as *Lr34res*. Further, we hypothesize that both contribute to a common disease resistance mechanism. Due to the different protein structures of Lr67res and Lr34res, they most probably do not share the same substrate. Therefore, we think that both Lr67res and Lr37res contribute to resistance by influencing distinct modules of a common pathway.

1.2 Altered expression of *Lr34res* in barley leads to quantitative disease resistance without negative pleiotropic effects

In barley, expression of *Lr34res* under its native wheat promoter leads to negative pleiotropic effects on growth vigour. Intensity of disease resistance and LTN correlates with expression levels of *Lr34res*. Altering *Lr34res* expression allows reducing negative pleiotropic effects while *Lr34res*-mediated disease resistance is maintained. This can be achieved by exploiting the interaction with *Lr34sus* on the transcriptional level in barley lines transgenic for both alleles (*Lr34res*x*Lr34sus*) (Chapter B) or the use of the pathogen-inducible *HvGer4c* promoter (Chapter C). Based on these two approaches, we could identify barley lines harboring *Lr34res* showing partial multi-pathogen disease resistance without impact on fitness costs. These lowered fitness costs in turn enable a high agronomical potential of our barley lines transgenic for *HvGer4c*::*Lr34res* or *Lr34res*x*Lr34sus* that provide an important base for breeding for durable disease resistance in barley.

1.3 Artificial alleles indicate that the potential repertoire of durable quantitative resistance genes is not exhausted by natural evolution

Lr34res is the only identified natural *Lr34* allele mediating disease resistance and it evolved from *Lr34sus* after domestication (Krattinger et al., 2013; Krattinger et al., 2009). Our results demonstrate that not all possible mutations in susceptible *Lr34* alleles leading to disease resistance have evolved naturally (Chapter B and D). We describe three artificial *Lr34* alleles (*M1*, *HAΔI545* and *HAΔM547*) mediating disease resistance similar to *Lr34res*. Given the relative short time in the context of evolution, we conclude that potentially more than the presented alleles would lead to disease resistance. Theoretically, these potential alleles could be artificially created by site-directed mutagenesis. The creation of additional quantitative resistance genes would enrich the diversity of the natural repertoire. Further, artificial quantitative resistance genes might show less negative pleiotropic effects compared to naturally evolved ones (discussed in Chapter D).

1.4 Lr34 variants mediating disease resistance or susceptibility show distinct protein levels

Finally, we found that all resistant Lr34 variants tested (HALr34res, HAΔI545 and HAΔM547) showed higher protein levels than susceptible ones (HALr34sus, HAΔF543 and HAΔF542/ΔF543). We modelled the protein structure of Lr34sus based on the human ABCG5/ABCG8 crystal structure. The model revealed that all deletions in Lr34sus (HAΔI545, HAΔF546 and HAΔM547) leading to disease resistance are located below this kink (towards the intracellular membrane layer), whereas all deletions not altering the susceptible phenotype (HAΔF543, HAΔF542/ΔF543) are located above the kink (towards the extracellular membrane layer). Therefore, we hypothesize that the different protein levels of resistant and susceptible Lr34 variants are caused by protein structure. The two most common mechanisms regulating protein level are degradation via phosphorylation or Endoplasmatic Reticulum Associated protein Degradation (ERAD) (Huttner and Strasser, 2012; Meusser et al., 2005). Phosphorylation requires phosphorylation sites accessible for cytosolic kinases. Our model showed that all amino acid polymorphisms tested were located in a

transmembrane helix. Therefore, we concluded that susceptible Lr34 variants might be rather and undergoing ERAD than are degraded via phosphorylation.

2. Outlook

2.1 The localisation of Lr34res will help to get more insight in the molecular function of Lr34res

So far, all full size ABCG transporters, for example ABCG40 (Kang et al., 2010) or ABCG25 (Park et al., 2016), are localised in the plasma membrane. Further, Lr67res that shows a similar phenotype as Lr34res, is also localised in the plasma membrane. Therefore, it is likely that also Lr34res is localised in the plasma membrane. However, a more detailed analysis of sub cellular and tissue localisation will be crucial to get more insight in the molecular function of Lr34res. Although many approaches regarding the localisation of Lr34res failed, it is worth to invest more effort in this issue. Further experiments would include subcellular protein fractionation of barley transgenic for *HALr34res* (Chapter D) using ultracentrifugation with the sugar gradient method (Geisler et al., 2003). In combination with organelle specific markers (Higuchi-Sanabria et al., 2016; Nelson et al., 2007) that allow co-localisation experiments, it could be identified in which membrane Lr34 is located. Further, barley transgenic for *HALr34res* could be used for immunolocalisation *in vivo*. Although the N-terminally HA-tag in barley *HALr34res* lines was detectable in Western Blot assays, it is not yet clear if the HA-tag is accessible for antibodies *in vivo*. A C-terminally HA-tagged Lr34 variant could assist in this regard. In general, C-terminal tagged Lr34 variants were not created so far. Therefore, a set of C-terminal tags could be added to Lr34. One possibility is the citrine tag, a variant of the Yellow Fluorescent Protein (YFP) which shows higher pH-stability than GFP (Griesbeck et al., 2001).

2.2 *Lr34res* is a valuable source for disease resistance breeding in different cereal species

Lr34res has been shown to mediate disease resistance without fitness costs in maize and durum wheat (Rinaldo et al., 2016; Sucher et al., 2017). Maize and durum wheat lines transgenic for *Lr34res* were investigated under glasshouse conditions. It would be interesting if maize and durum plants transgenic for *Lr34res* show disease resistance without negative pleiotropic effects on growth vigour under field conditions. If this could be confirmed, *Lr34res* might provide a source for quantitative disease resistance in breeding of maize and durum wheat.

In rice and *Sorghum*, disease resistance mediated by *Lr34res* without negative pleiotropic effects was either found in one single transformation event or only at seedling stage, respectively (Krattinger et al., 2016; Schnippenkoetter et al., 2017). In this case, negative pleiotropic effects on growth vigour could hinder the direct agronomical application of the rice and sorghum lines transgenic for Lr34res under the native wheat promoter. Therefore, similar strategies to avoid negative pleiotropic effects as presented for barley (Chapter B and C) could be applied in rice and *Sorghum*. The barley lines showing disease resistance without negative pleiotropic effects on growth vigour (Chapter B and C) can be tested in large field trials or used as parents to cross *Lr34res* in elite barley cultivars.

In wheat one possibility to exploit *Lr34res*-mediated resistance in elite cultivars would be to modify *Lr34sus* alleles present in wheat cultivars to *Lr34res*-alleles by genome editing of the loci described in Chapter D. In wheat this is only appropriate if time-consuming (back-) crosses are undesired. In addition, genotype dependant transformation efficiencies might hinder this approach. Therefore, in wheat, the transfer of *Lr34res* in elite cultivars by classical crossing seems more appropriate.

2.3 CRISPR/Cas9 might be used to introgress quantitative disease resistance in cereal crops while circumventing the GMO debate

Wheat *Lr34res* has been shown to confer disease resistance in barley (Risk et al., 2013), rice (Krattinger et al., 2016), maize (Sucher et al., 2017), durum wheat (Rinaldo et al., 2016) and *Sorghum* (Schnippenkoetter et al., 2017). The most obvious strategy to introduce *Lr34res* in modern breeding programs for disease resistance would be to use the already created *Lr34res* transgenic lines as source for crossings with elite cultivars. Regarding social-political issues the use of crop cultivars transgenic for *Lr34res* seems most promising in maize (USA) where cultivation of genetically modified organisms (GMOs) is possible. Lack of acceptance of GMOs in Europe (Lamichhane et al., 2016) will most probably hinder the use of transgenic plants. Therefore, in Europe other strategies to include quantitative disease resistance in breeding are needed. One idea would be to apply the genome editing method known as CRISPR/Cas9 that is still not considered to create GMOs (Jinek et al., 2012; Ochiai, 2015). The principle is that Cas9 (CRISPR-associated protein 9) is guided to the DNA sequenced of interest by a 20 base pair sequence guide RNA (sgRNA). Cas9 introduces a double strand break which is either repaired by non-homeologous end joining (NHEJ) or by homeology-directed repair (HDR) if a (addable) oligo template is present. However, NHEJ is imprecise and therefore used to create knock out mutants by indroducing a frameshift or a premature stop codon. In contrast to NHEJ, HDR is precise and used for targeted sequence changes (Jinek et al., 2012; Mali et al., 2013; Symington and Gautier, 2011). Using different sgRNAs, different simultaneous mutations are possible, a method called multiplex genome engineering (Cong et al., 2013). Elaborated protocols for genome editing of rice and wheat already exist (Liang et al., 2017; Yang, 2017). In the case of *Lr34*, susceptible *Lr34* alleles could be changed to resistant alleles by the insertion of deletions encoding for amino acid deletions in the region of F546.

2.4 Possibilities and limitations of genome-editing by CRISPR/Cas9

One promising advantage of genome editing is that the mutations can be locally targeted in the genome. This might solve problems faced in classical breeding or conventional transformation protocols, where a gene of interest cannot be separated from linkage drag (negative effect from neighbouring genes or sequences). On the other hand off-target effects of the RNA-guided endonucleases have also been reported (Cho et al., 2014). However, strategies to reduce CRISPR/Cas9-associated off-target effects to non-detectable levels have also been shown recently (Kleinstiver et al., 2016). The simplest CRISPR/Cas9 approach is to create knock out mutants. Therefore, the possibly best strategy to enhance disease resistance by genome editing is to knock out recessive disease resistance genes. As genome editing by CRISPR/Cas9 allows creating mutations in homoeologous genes simultaneously, this method promises a huge potential for the fast creation of new disease resistant polyploid and genetically complex crop cultivars. One example of which this method has already been reported is the simultaneous knock out of all three barley *Mlo* homeologs of wheat resulting in resistance against powdery mildew (Wang et al., 2014). Using similar approaches as described above, recessive resistance genes as rice *Pi21* could be converted to *pi21* to gain durable resistance against rice blast (Fukuoka et al., 2009). This strategy could also work for other susceptibility genes as *TsnI* and *SnnI* (Chu et al., 2010) to mediate disease resistance against *Stagonospora nodorum* blotch in wheat or for *Xa24* to mediate resistance against the bacterial blight caused by *Xoo* in rice (Busungu et al., 2016).

The biggest limitation of genome editing by CRISPR/Cas9 is that the sequence information of the gene of interest has to be known. Otherwise, targeted mutations are impossible. This is especially a problem as many resistance genes are not yet cloned. Even a resistance gene as *Lr34* has been cloned, one needs to know which exact sequence changes lead to altered phenotypes. Genome editing per se is only a technique to create new gene mutations that can be used to test genetic

hypothesises *in vivo*. As CRISPR/Cas9 still requires a transformation step, it is in question if this method is really more efficient than classical site-directed mutagenesis followed by transformation.

2.5 Artificially generated quantitative resistance could lead to an enormous progress in crop breeding

Based on the knowledge of our artificial *Lr34* alleles we propose to test if other mutations in the region of residue F546 of Lr34sus lead to disease resistance. The results might reveal critical protein structures needed to convert susceptible Lr34 variants into resistant ones. Ideally, a possibility to convert the susceptible orthologs to resistant ones could be found. So far, we deleted only methionine, isoleucine and phenylalanine(s). The deletions leading to disease resistance (ΔI545 and ΔM547) involved both nonpolar hydrophobic amino acids that show similar molecular structures than phenylalanine. Maybe therefore, the deletions of I545 or M547 had the same effect. It would be interesting to exchange I545, F546 or M547 by a glycine. Perhaps the smallest amino acid glycine could phenocopy a deletion, although its polar, hydrophilic character might cause severe changes in the protein structure.

Interestingly the two cloned quantitative resistance genes *Lr34res* and *Lr67res* encode for transporters. Therefore, we speculate, that other transporter genes could have the potential to convert quantitative disease resistance. Additional transporters could also contribute to the common disease resistance mechanism of Lr34 and Lr67 (hypothesized in conclusions). As a first step one could create artificial alleles of Lr67 analogously to our artificial Lr34 alleles. Lr67res evolved from Lr67sus by the two amino acid polymorphisms G144R and V387L. Therefore, amino acid polymorphisms around G144 or V387 in Lr67sus could be tested for its ability to mediate disease resistance. Together with results from Lr34, critical amino acid sequences needed to mediate disease resistance could be identified. Later, one could look for homeologous sequences in other transporters and mutate them analogously. If several transporters could be identified to contribute to quantitative resistance, the understanding of the quantitative resistance-causing mechanism

would be ameliorated. In addition, it would allow the introduction of artificial quantitative resistance in breeding. This in turn could lead to an enormous progress in modern cereal crop breeding for durable disease resistance.

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